Investigating Late Stage Biopharmaceutical Product Loss Using Novel Analytical and Process Technology

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Submitted to the MIT Sloan School of Management and the Biological Engineering Department in Partial Fulfillment of the Requirements for the Degrees of

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ABSTRACT

The biopharmaceutical industry uses recombinant protein technologies to provide novel therapeutics to patients around the world. These technologies have presented exciting opportunities for breakthrough medical treatments while creating a host of challenges in the discovery, development and manufacture of these products. Protein aggregation is one of the challenges currently limiting the ability to bring new biopharmaceutical products into the market and to manufacture existing commercial products. The mechanisms of aggregation and subsequent particle formation are highly complex, incompletely understood, and difficult to measure quantitatively with currently available analytical tools. Aggregates, and their effect on product appearance, may compromise value to the patient (bioavailability, dose, therapeutic activity and immunogenicity) as well as value to the company (yield loss and performance in a competitive marketplace) and are therefore tightly regulated.

This thesis is intended to explore the problem of protein particles through two main avenues: meeting current regulatory criteria and influencing future regulation. Process changes, analytical characterization, and organizational improvements are each addressed to achieve that goal. An experiment was designed and completed to jointly examine (1) changes to manufacturing processes using novel filtration applications intended to reduce or remove protein particles from solution and (2) analytical tools for improved characterization. Organizational dynamics and resource allocation add an extra layer of complexity and are discussed in relation to leveraging knowledge regarding particles. Additionally, three objectives are established to influence the direction of future regulation: the need for improved characterization, industry collaboration and a healthy interface with regulatory bodies.

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1. Introduction

Biotechnological medicines, first introduced in 1982, were expected to revolutionize the pharmaceutical industry. With a lower toxicity and a more predictable behavior in vivo, these medicines could potentially reach the market faster and benefit more patients than the traditional chemical entities available at the time. However, the advent of new technologies brought with it a host of novel challenges that curb the rate of industry growth.

Up to 96% of all drug candidates in trials are abandoned during preclinical or clinical development, often because of low solubility or intrinsic propensity to aggregate irreversibly (Caldwell, 2001, Chi, 2003). Irreversible aggregation presents one of the most difficult obstacles in the development of high concentration protein therapeutics due to a lack of clear understanding of the protein aggregation mechanistic process and the potential safety impact of protein particles. Aggregates and their effect on product appearance may compromise value to the patient (bioavailability, dose, therapeutic activity and immunogenicity) as well as value to the company (performance in a competitive marketplace). Since it is the paramount responsibility of biotech companies and regulatory bodies to provide the best guarantee of safety and efficacy to patients, the problems surrounding protein aggregation must be better understood. To achieve that end, academic institutions, biotech firms and regulatory authorities must collaborate to establish the scientific background needed to address the aggregation problem to bring promising new biopharmaceuticals to market for the benefit of patients and society. This thesis attempts to be one small piece of that effort, by focusing first on a novel approach to reduce protein particles to meet current regulatory policies and secondly to influence future policies through improved standardization and process characterization as prescribed in the International Conference on Harmonization (ICH) Guideline no Q8 (ICH Expert Working Group, 2007).

1.1. Project Drivers and Overview

Amgen, Inc. is currently the largest biotech company (by revenue) and one of the most respected firms in the industry. Driven by the mission "To Serve Patients," Amgen strives to develop innovative therapies for serious illnesses. Focusing on therapeutic proteins, Amgen is

continuously challenged by the complexity of developing and manufacturing such products. One possible challenge in bringing these products to market is protein aggregation and the formation of aggregates into protein particles, some of which are visible to customers. Not only does protein aggregation limit the ability to bring novel molecules out of Research and Development, but it can also present significant challenges to products that have been successfully commercialized. Protein aggregation is highly complex because the mechanism is incompletely understood and while the problem is difficult to measure quantitatively it is tightly regulated under limited information. Current regulation states that products labeled as "clear", rolling off of the manufacturing line and exhibiting detectable levels of visible protein aggregation must be scrapped and result in lost material, as rework is not an option. This yield loss can impact two of Amgen's main goals: serving every patient, every time and delivering value to shareholders.

This work attempts to explore the problem of visible protein particles through two main avenues. It is divided into work looking to meet current regulatory criteria and that looking to influence future regulation. In depth attention is given three main elements to address the current environment: process changes, analytical characterization and organizational improvements. An experiment was designed to jointly examine both changes to manufacturing processes using novel filtration applications intended to reduce or remove protein particles from solution and analytical tools intended to improve characterization. Organizational dynamics and resource allocation present an extra layer of complexity and are discussed in relation to leveraging knowledge regarding particles. In influencing the direction of regulation, three objectives are discussed: the need for improved characterization, industry collaboration and a healthy interface with regulatory bodies.

1.2. Problem Statement

As the biopharmaceutical industry continues to advance, it is the responsibility of regulatory bodies to update regulations based on the most recent good science and the responsibility of manufacturers to meet those regulations. Novel therapeutics, particularly high concentration proteins, have a propensity to form visible proteinacious particles. These particles meaningfully differ in nature from foreign visible particles and should therefore be regulated in a context appropriate to their science starting from early development through the post marketing

experience. Addressing proteinacious particles is a high priority for biopharmaceutical manufacturers requiring superior organizational management to enable the application of best practices across platforms and products.

1.3. Thesis Overview

This thesis is designed to add to the body of knowledge regarding the risk mitigation of protein particles to the biotech industry through two broad categories; meeting new ICH regulatory standards and influencing future regulation pertaining to the application of new standards to future drug candidates. In meeting this end, six chapters have been developed:

Chapter 2 provides background information to level set the reader with background relevant to protein particles and drug product regulation. It includes a basic overview of proteins and a general understanding of protein aggregation followed by both historical and current regulatory measurements and acceptability criteria. It also provides context around the extensive impact protein particles could have on process characterization at a biotech firm and on the patients it serves.

Chapter 3 explores potential solutions to the problem in a framework created to highlight particle relevant processes in the commercial development of a biological pharmaceutical. A literature review of current academic and industry research is provided to arm the reader with a concise understanding of distributed efforts and to highlight the importance of a holistic approach. Additionally, it explores the application of organizational changes intended to leverage knowledge management.

Chapter 4 discusses the importance of comprehensive analytical characterization and briefly reviews new technologies and method development.

Chapter 5 investigates applications in particle reduction through an experiment using novel process and analytical technology.

Chapter 6 synthesizes the individual results presented in the thesis into a cohesive recommendation for a proactive and holistic approach to characterize and mitigate protein particles.

2. Background

Protein aggregates present a formidable challenge for biopharmaceutical companies and are one of the many road blocks to fully realizing the potential of protein therapeutics. Chapter two is intended to provide the reader with background information to understand the complexity of the problem and the challenge to realize a pragmatic solution. The following sections will deal with protein particles from a very general base: proteins and their use as therapeutics, a brief introduction to protein particles, their measurement and regulation, followed by a short summation of the considerable impact of protein particles on companies producing protein therapeutic products.

2.1. Protein Chemistry

Proteins, involved in almost all biological activities, are the foundation for therapeutic biopharmaceutical products. Deoxyribonucleic acid (DNA) both replicates itself and codes for the production of Ribonucleic acid (RNA) that then codes for the synthesis of proteins. Constructed from their component parts (amino acids), proteins are the final product in the central dogma of molecular biology. Figure 1 describes replication of DNA, transcription from DNA to RNA and translation from RNA to protein.

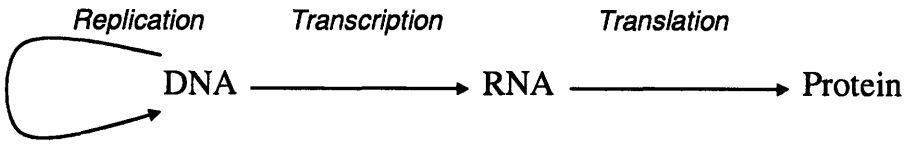

Figure 1: Molecular Biology Central Dogma

Under the proper conditions, proteins are self-assembling and spontaneously fold into their native conformations in a matter of seconds as determined by their primary structure or sequence of amino acids. Proteins are flexible and fluctuating molecules in which molecular dynamics simulations indicate that native proteins structures each consist of a large number of closely related and rapidly interconverting conformational substates of nearly equal stabilities (Voet,

2004). The flexibility and sensitivity of these molecules make them both difficult to characterize and at high risk of degradation and aggregation into a variety of unpredictable conformations.

2.2. Protein Therapeutics

Recombinant DNA techniques and similar applied biology technological breakthroughs have made it possible to create and manufacture human proteins characterized as biopharmaceutics and generally referred to as protein therapeutics. Protein therapeutics are capable of treating diseases when properly used and differ significantly from traditional small-molecule pharmaceuticals. Small-molecule therapies are typically 20-100 atoms in size, homogenous in purity and created through organic synthesis. Protein therapeutics (large molecules) are instead much larger, ranging from 200-50,000 atoms, heterogeneous, produced biologically and have a number of advantages over small-molecule drugs. Proteins serve a highly specific and complex set of functions often with less potential to interfere with normal biological processes and because the body naturally produces many of these proteins, they are often well tolerated and less likely to illicit an immune response (Leader, 2008). Therapeutic proteins are organized below by Leader (2008) based on their function and therapeutic action.

Protein therapeutics with enzymatic or regulatory activity
- Replacing a protein that is deficient or abnormal
- Augmenting an existing pathway
- Providing a novel function or activity

Protein therapeutics with special targeting activity
- Interfering with a molecule or organism
- Delivering other compounds or proteins

Protein vaccines
- Protecting against a deleterious foreign agent
- Treating an autoimmune disease
- Treating cancer

Produced biologically in bacterial or mammalian cells, proteins are harvested and purified by filtration or fractionation using characteristics such as solubility, ionic charge, polarity, molecular size and binding specificity. The purified protein is then formulated into a stable solution safe for delivery to the patient. Because proteins are sensitive to digestive enzymes in the stomach, they must be delivered in an aqueous parenteral form including intramuscular,

subcutaneous or intravenous injection. The final drug product is defined as both the protein in a formulated solution (lyophilized or aqueous) and the delivery system (vial or pre-filled syringe).

2.3. Generic Protein Therapeutic Manufacturing Process

Biopharmaceutical manufacturing process differs greatly from traditional chemical pharmaceuticals and is reviewed below in Figure 2 (Adapted from Walsh, 1999).

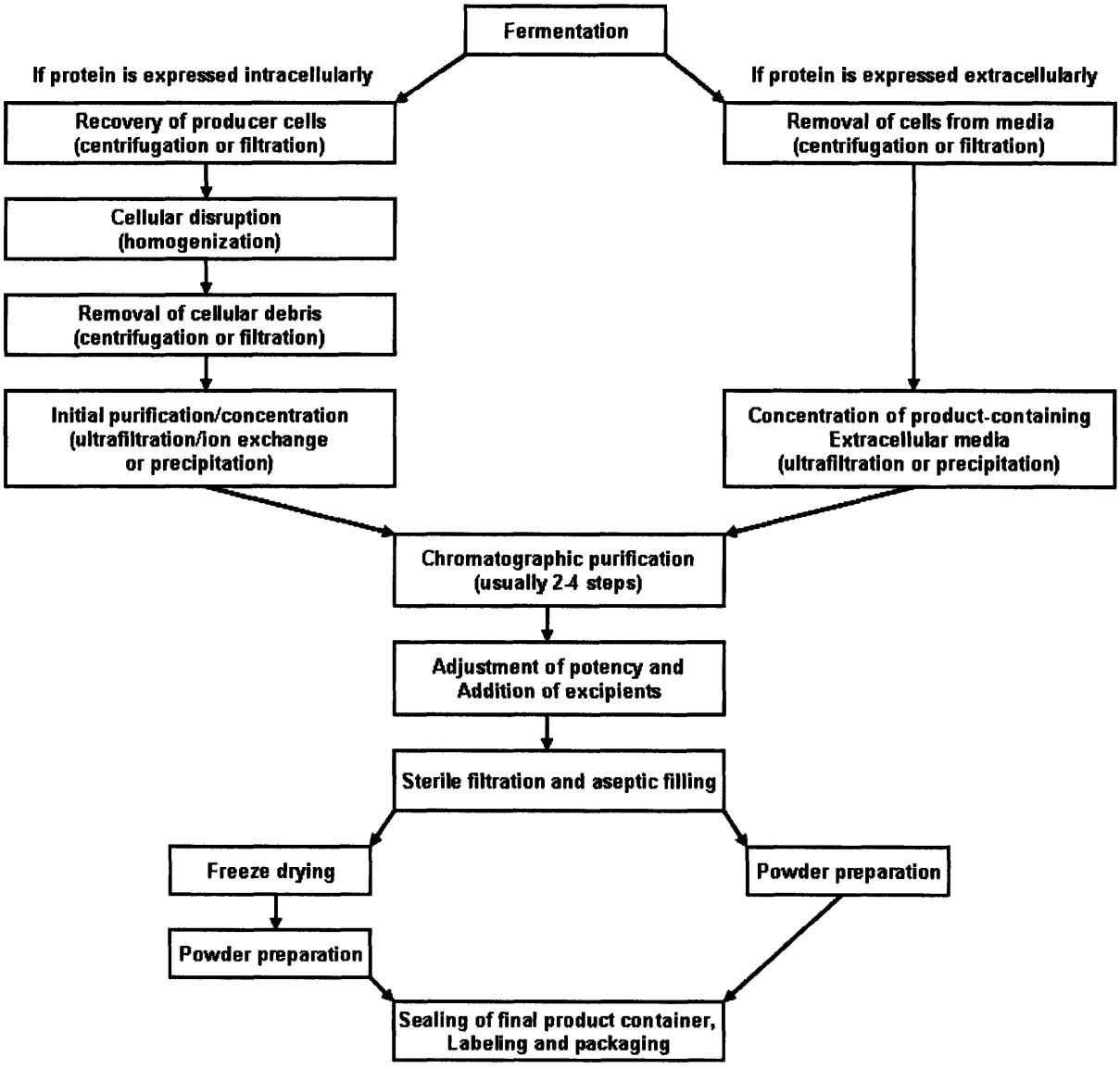

Figure 2: Biopharmaceutical Manufacturing Process Overview

The Formulation, Fill, Finish processes are especially important for particulate considerations and are covered in more detail in Figure 3 (Rathore, 2008).

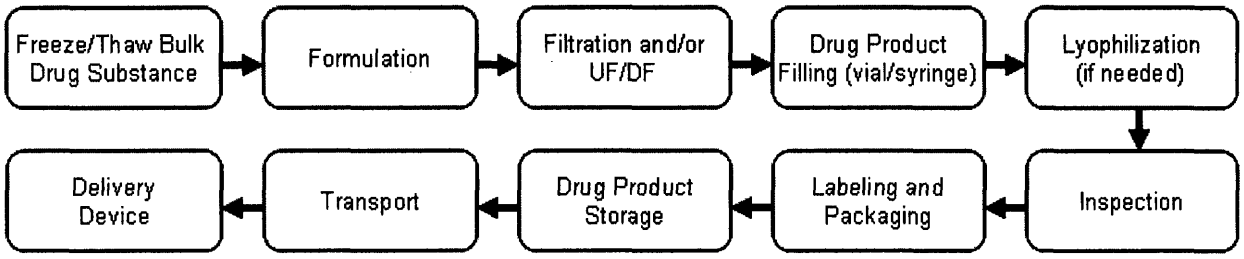

Figure 3: Formulation, Fill, Finish Operations

2.4. Particulate Matter in Therapeutic Parenterals

Particulate matter presents a host of problems in the manufacturing of therapeutic proteins. Sub-visible and visible Particles range in material type (i.e. foreign or proteinacious), size, color, shape, buoyancy, and exist in range of three orders of magnitude (1 to 1000 μm). The range from 0.01 to 1 μm represents semi-soluble aggregates which serve as pre-cursers or nuclei to the larger particles. Because parenterals are injected into patients, acceptable levels of particles are regulated by the FDA (to be further explained in Chapter 2.5). Today, the elimination of particles from injectable solutions is heavily dependent on the use of filters (Barber, 2000). However, it is thought to be virtually impossible to remove every vestige of unwanted particulate matter from an injectable solution. Furthermore, it becomes increasingly expensive to remove particulate matter as the size of the particulate decreases (Barber, 2000).Table 1 illustrates the variety of contaminants and their sizes reported in parenteral solutions (Groves, 1973).

Table 1: Approximate Sizes of Some Contaminants Reported in Intravenous Solutions

Contaminant	Size Range (μm)
Insect parts	20-1,000
Glass fragments	1-1000
Rubber fragments	1-500
Trichomes	10-100
Metal particles	1-100
Cellulose fibers	1-100
Lubricating and machine oil	1-100
Plastic fragments	1-100
Starch	5-50
Fungi	5-10
Zinc oxide	1-10
Calcium carbonate	1-10
Plasticizer droplets	1-10
Silicone oil droplets	0.01-10
Carbon black	1-5
Clay	1-5
Diatomaceous earth	1-5
Talc	1-5
Bacterial fragments	0.1-5
Viruses	0.05-0.1

The types, sizes, and numbers of particles in solution are particularly important when understanding patient safety and efficacy. While some particles may have a serious adverse effect on the patient (e.g. blocking the pulmonary micro-capillary network or by an immune response), others may only be considered a cosmetic defect. In such a case, the presence of particles may affect customer perception of a protein therapeutic and impact its financial performance in a competitive marketplace. There are two types of particles that find their way into the therapeutic treatments; foreign particles that are not related to process contact materials (extrinsic particles) and particles that are related to the process environment (intrinsic or proteinacious).Table 1 illustrates mostly extrinsic particle types, which are arguably less difficult

to find and characterize. Proteinacious or intrinsic particles are a function of protein aggregation and are the focus of this work.

2.4.1. Extrinsic Particles

Extrinsic particles are those that enter the product or its container during manufacturing processes. They typically include rubber, metal, or plastic coming from the manufacturing environment, such as paint chips or dust (Barber, 2000; E. Freund, personal communication, 2008). Although foreign particles can be introduced at multiple steps in the manufacturing process, filtration and purification processes are in place to remove such irregularities, making the final transfer of product to the container most susceptible. Due to the potentially serious adverse effects extrinsic particles on patient safety, the FDA has developed current Good Manufacturing Processes (cGMPs) in which the control of particles in pharmaceutical processing is one element. Key points of cGMP controls that can impact particulate matter control include: control and/or classification of manufacturing areas, positive pressure environments, airflow patterns and airflow velocity, filtered air, particle count monitoring, suitable premises, equipment and materials, trained personnel and adequate transport and storage (Barber, 2000). Although extremely important to consider, extrinsic particles are not the focus of this work and are only discussed with respect to intrinsic particles.

2.4.2. Intrinsic Particles

Proteinacious particles, more often called protein aggregates, are a group of particles held together by strong atomic or molecular forces (Barber, 2000). Aggregation is generally considered the process by which protein aggregates with a secondary structures at the monomer scale are created that differ significantly from the dominant (therapeutic) structures in the native state (Roberts, 2007). Aggregate sizes cover a range from small oligomers to visible "snow globe" like precipitates. Generally only the smaller species can be reversed (Philo, 2006). While the kinetics of protein aggregation is generally poorly understood, its affects are critical to human health, product shelf life and ultimately the success of therapeutic products, (Cleland, 1993; Roberts, 2003) which explains the importance of this challenge.

Aggregation is thought to proceed from a native monomeric soluble protein, through the formation of aggregation-competent species, followed by association reactions to form non-native protein-protein contacts, resulting in the formation of higher molecular weight aggregates (Roberts, 2003; Chi, 2003). Figure 4 depicts a simplistic scheme of the reactions that can occur during initial aggregation of a protein where protein N is the native protein, N* is the aggregation competent species, and A_i is an aggregate with subscript i denoting is assembly state (Chi, 2003).

$$N \leftrightarrow N^*$$

$$N^* + N^* \rightarrow A_2$$

$$N^* + A_2 \rightarrow A_3$$

$$N^* + A_3 \rightarrow A_4$$

$$\vdots$$

$$N^* + A_{m-1} \rightarrow A_m$$

$$A_2 + A_2 \rightarrow A_4$$

$$A_2 + A_3 \rightarrow A_5$$

$$\vdots$$

$$A_2 + A_{m-2} \rightarrow A_m$$

$$\vdots$$

$$A_i + A_j \rightarrow A_{i+j}$$

$$\vdots$$

Figure 4: Aggregate Formation Schematic

Often divided into "soluble" and "insoluble" or "reversible" and "irreversible" categories, there is unfortunately no uniform terminology for aggregate sizes or types (Philo, 2006). It is not entirely clear when an aggregate transitions from an "oligomer" to a "particle," however, aggregates are generally understood to exist along some dynamic range with the potential to move in and out of certain states depending on factors such as solvent components, excipients, organic modifiers, pH, temperature and time in addition to processing constraints (Wang, 2004; Philo, 2006). Variations in these factors amongst therapeutic proteins are only one of the challenges in studying proteinacious particles. However, characterizing protein aggregation is of

fundamental importance to the manufacturing, storage, and delivery of biopharmaceutical products.

2.4.2.1. *Challenges of Formation Kinetics and the Dynamic Range*

Roberts (2007), Figure 5 below, depicts protein folding in its simplest form and the multi stage process of non-native aggregation as currently understood. Non-native aggregation is generally considered to be any process which creates protein aggregates with secondary structures at the monomer scale that are significantly different from the dominant structures in the native state and for which these structures are at least putatively stability or influence the morphology, size, toxicity, or material properties of the resulting aggregates (Cox, 2005; Rosenberg, 2006). Monomers that are incompletely folded or non-native are most often implicated as the reactive precursors that subsequently assemble to form higher molecular weight aggregates (Chi, 2003; Dobson, 2004). The portrayal of native polymerization in Figure 5 does not take into account conformational changes or aggregate-aggregate association, but represents the historical basis for a number of kinetic models of protein aggregation (Roberts, 2007).

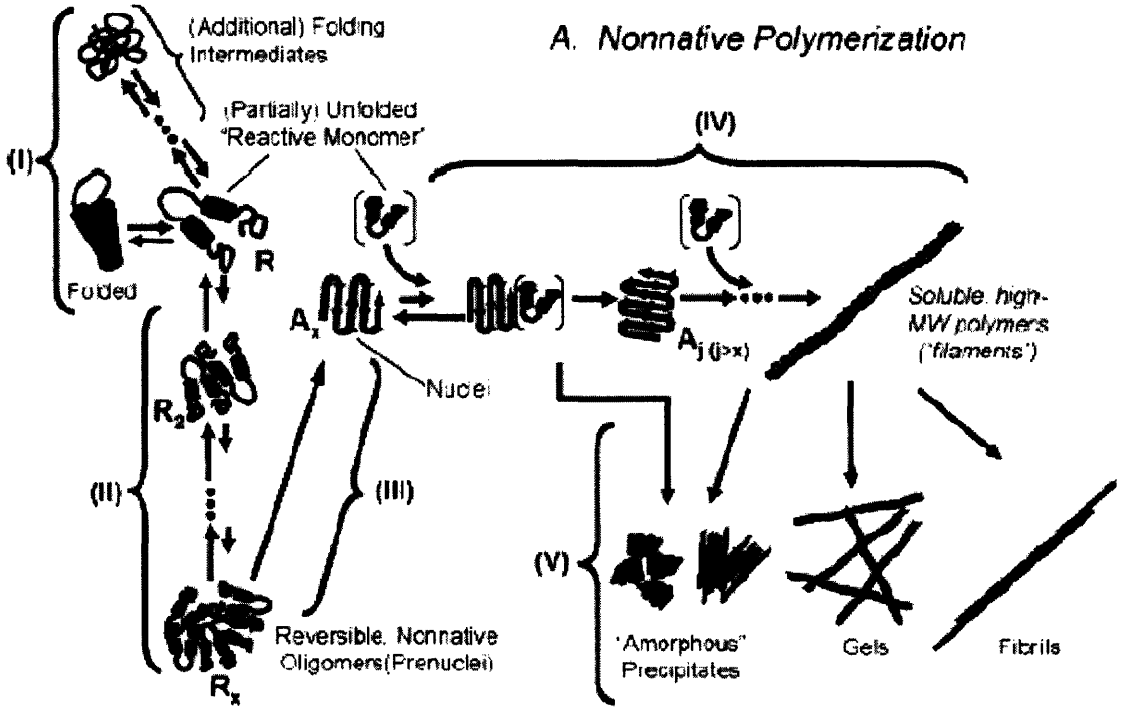

Figure 5: Non-native Protein Polymerization

In his review, Roberts (2007) also notes that "*many if not all of the intermediates depicted in stages II, III, and potentially stage IV are often difficult if not impossible to reliably quantify or even detect experimentally...*" The inability of most available experimental methods and analytical tools to detect and quantify key intermediates in non-native aggregation (Philo, 2006) or even to distinguish their structural features from those of upstream and downstream species in the mechanism remains a principle challenge (Roberts, 2007). Most methods are used for relative qualitative analysis and describe the aggregates using a diverse assortment of information, often not overlapping with other analytical tools or informative for all practical purposes. Because aggregates exist in a dynamic range, in conditions which are exceptionally difficult to exactly replicate, it is not surprising that the population distributions of these aggregates are so difficult to measure.

2.5. Regulation of Protein Therapeutics

As the official public standards-setting authority for healthcare products, including protein therapeutics, manufactured and sold in the United States, the U.S. Pharmacopeia sets standards for the quality of these products. After the FDA has approved a pharmaceutical product, the USP works with manufacturers to develop tests, procedures and acceptance criteria standards that are legally enforceable by the FDA. International manufacturers who make products for sale in the U.S. must also comply with USP standards and compendial methods. The purpose of the USP is to protect the patient by ensuring that drugs administered are of the highest quality with regard to purity, efficacy and safety (Barber, 2000; U.S. Pharmacopeia, 2008). Furthermore, determining testing standards and acceptance criteria of particulate matter is one of the many ways the USP ensures patient safety. Similar international organizations exist for products sold globally which include the European, British, and Japanese Pharmacopeias which each provide a listing of a wide range of active substances and excipients used to prepare pharmaceutical products.

2.5.1. Regulation of Particulates in Parenterals

The solution product cleanliness requirements of the pharmaceutical industry (U.S. Pharmacopeia [USP] <788>) were historically derived from the human body and the physical

dimensions of its vasculature (Barber, 2000). According to the USP, particulate matter is defined as mobile, randomly-sourced, extraneous substances, other than gas bubbles, that cannot be quantified by chemical analysis due to the small amount of material that it represents and to its heterogeneous composition (USP, 2006). Assuming that the smallest capillary of the human body has a diameter of 7 μm (the diameter of a red blood cell) the particulate matter of concern is considered to be that larger than 10 μm (USP) and often more conservatively 5 μm (European Pharmacopeia) (Barber, 2000). Although there is currently no absolute definition of "visible", as it is a probabilistic determination (Knapp, 1996), for the purposes of this discussion, subvisible particles are defined as <150μm based on the upper limits of the light obscuration instrument used to measure them (to be discussed in more detail under the Analytical Techniques section in Chapter 4). The tests outlined by regulation to measure particles are performed for the purpose of enumerating subvisible extraneous particles within a specific size range. Current USP limits for extraneous particles classify a failed product if it exceeds a determined concentration of particles in solution. Table 2 outlines the USP guidelines for a concentration of particulates of varying sizes in solution. The guidelines are followed based on total volume and sampling (individual or pooled) for particulate testing. According to both techniques (the light obscuration and microscopic method), when samples are pooled solutions for parenteral infusion or solutions for injection are supplied in containers with a nominal content of more than 100 mL, the Particles per mL rule is applied. When solutions for parenteral infusion or solutions for injection are supplied in containers with a nominal content of less than 100 mL, the Average Particles per Container rule is applied.

Table 2: USP <788> Particulate Matter Limit Classification

	Average Particles per mL		Average Particles per Container	
	≥10 μm	≥25 μm	≥10 μm	≥25 μm
Light Obscuration Method	20	3	6,000	6000
Microscopic Method	12	2	3,000	300

In addition to interfering with arterial passage, protein aggregates pose a risk in terms of generation of immune responses to the therapeutic protein product. Of principal concern are those immune responses associated with adverse clinical effects: creation of a neutralizing

antibody that inhibits the efficacy of the product, cross reactive neutralization of an endogenous protein counterpart, or severe immediate hypersensitivity responses such as anaphylaxis (Rosenberg, 2006). For example, early commercially available Human Serum Albumin or pasteurized plasma solutions containing 5-15% aggregates caused severe anaphylactic responses in some patients (Ring, 1979). The extent to which these responses impact therapy is determined by multiple factors, but because protein aggregates can in certain cases induce immune responses, manufacturers should employ several measurement methods for robust assessment of protein aggregates in products.

2.5.2. Historical Measurement of Particulates in Parenterals

The two basic approaches for determining particle size distribution and concentration in injectable solutions are the microscopic technique and the automatic particle count devices using light obscuration. The microscopic technique is typically used to find information on particle shape and source identification. However, the Light Obscuration technique is considered to be more reproducible, faster, and robust in determining quantitation (DiGrado, 1970). Each of these techniques, including their capabilities and limitations, are briefly reviewed below.

2.5.2.1. Light Obscuration

The Light Obscuration (LO) Liquid Particle Counting System relies on light obscuration to detect and quantify the number of particles and their size range in a given test sample. Introduced in 1969, the first and current instruments operate on the interaction of an intense collimated and focused beam of light with a particle suspended in a liquid medium (Barber, 2000). Particles are forced to pass through a narrow view volume between the light and a photodiode detector. The presence of particles is measured by the variation in light intensity which is then translated to a voltage signal. As a result, the machine must be calibrated to an ideal standard particle, such as high contrast polystyrene microspheres of a certain size, which greatly differ from the properties of a protein particle.

Capable of detecting particles as small as 2μm, optical effects and electrical and mechanical functions of the system have inherent limitations. Because the technology has made very few changes over time, Barber's (2000) observations are still highly relevant and are summarized

below as core concerns in relation to pharmaceutical particle counting: "*The variation of light scattering with particle transparency, refractive index, and size has serious implications for the sizing accuracy of counting when particles of different transparencies, sizes, colors, and surface textures are present in the sample.*" He elaborates that the primary mechanical limitations are optical or physical coincidence: "*In this case, multiple particles are simultaneously present in the view volume or sensing zone of a sensor and are sensed as one particle. This results in the collection of an artificial count at a size generally corresponding to the cumulative cross-sectional area of the particles being considered.*" Despite these limitations, the technology and methods have been accepted by regulatory bodies and remain established.

Additional limitations of the mechanics and technology may include:

- Refractive index dependency of the LO measurement
- Validation and count accuracy
- Resolution effects
- Sources of erroneous count data
- Particle size and shape bias
- Air or gas bubbles
- Excessive degassing
- Variability due to sampling effects
- Issues relating to nonaqueous vesicles, color and viscosity
- Interferences from subcountable-sized particles
- Intermittent instrument problems

2.5.2.2. Manual Light Microscopy

The manual microscopy test is intended to provide a qualitative method for identifying particles that may be present in a solution and for determining their characteristics. This test enumerates subvisible, essentially solid, particulate matter in pharmaceutical products on a per-volume or per-container basis, after collection on a microporous membrane filter (Barber, 2000). The method is complex and requires the use of a compound binocular microscope, two illuminators, a filtration apparatus and a particle controlled laminar airflow enclosure. The sample is essentially vacuum filtered through the apparatus and all filtrate is collected on the surface of the membrane having a minimum diameter of about 21mm and porosity of 1.0μm or finer. The membrane is illuminated and investigated under the specified microscope. Particles are then counted and characterized accordingly.

The subjectivity of the test means that the key to the effective application of the light microscope in particle analysis is the availability of an experienced analyst. It requires only basic training and although it takes more time than does the application of liquid particle counting by LO, it is not subject to false counts due to air bubbles or artifacts due to immiscible liquids. However, disadvantages include low reproducibility between technicians, time consuming ultra cleaning of equipment, low throughput microscopic counting, rupturing of membranes and wrinkling of dried membranes (DiGrado, 1970; Barber, 2000).

2.6. Business Impact

Beyond the considerable resources invested in the research and development of potential therapeutics and the opportunity cost of the specific SKU's (stock keeping units) or drug product presentations that fail to make it to the market due to particle issues, protein particles have an additional impact on the bottom line. Commercialized products on the market may experience protein particle problems that generate complaints, reduce the yield of finished goods, affect the sales of the therapeutic in a competitive marketplace and pose a costly risk of noncompliance with a regulatory body. Commercial products afford less flexibility for change and continuous improvement than others in development as their complete processes are established end to end and approved by the FDA. The expense of changes made to these processes can often outweigh the potential improvements and make experimentation within the process nearly impossible. In the future, the ICH Quality by Design (QbD) principles are expected to offer greater flexibility, provided that more characterization and a greater mechanistic understanding of particle formation is achieved.

2.6.1. Yield Loss

One of the final steps in releasing finished product includes both subvisible and visible inspection (manual or automated depending on the container and product type) for foreign matter and cosmetic defects. In the case of manual visible inspection, certified inspectors hold the finished goods, vials or syringes, up to a defined fluorescent light against a black and white background and look for defined defects. Cosmetic defects could include a chipped vial or a stopper that is not sealed or crimped correctly. However, particulate defects are especially

challenging because it is not always as simple for inspectors to make a defect classification decision. Products that should be "particulate free" include freedom from both foreign and native (protein-like) particles. Any product that does not meet the particle requirements are scrapped without the potential for rework. An acceptable limit for particulates is determined for a product on a per container or per lot basis using statistical sampling methodology. A defined fraction of the whole lot may contain higher than expected visible particle levels as a function of the inspection sampling, and if the lot surpasses the limit, an investigation will ensue including forensic particle identification. If atypical particles are present throughout the batch, the whole lot may fail to be released. If a product is labeled (with FDA approval) as "may contain particles", meaning that it is a expected phenomena and known to pose no safety risk, inspectors must then be capable of identifying the particles as proteinacious or foreign (visible extrinsic particles are not permitted) and within an acceptable limit before the lot can be passed. Finished products that do not meet the appearance specifications are scrapped. Finished product is obviously the most valuable and therefore losses have the greatest relative impact on the Cost of Goods Sold (COGS).

2.6.2. Competitive Marketplace and Perceived Quality

Biotechnology companies will soon face the challenge of generic-like competition in the marketplace with follow-on biologics. Relatively new to the market, biological products in the initial pipeline have been protected by strong patents and therefore biotech companies have not had to face product competition typical of most industries. Follow-on biologics or biosimilars are the generic version of biological products and refer to a protein therapeutic that is comparable to, but not the same as, a previously approved therapeutic. Although the patents for some blockbuster biologics have already expired, the production of biosimilars has been stifled by the Food and Drug Administration's decision for biosimilar approvals. Amongst the industry, the argument continues as to whether it is possible to safely copy a complex biological pharmaceutical, like a conventional drug, without additional clinical trials to prove safety and efficacy. The magnitude of required clinical trials will impact the cost of production and therefore the ability of biosimilar products to compete with precursor original product. The recent approval and production of biosimilar products in Europe has confirmed these concerns. In April of 2006, the European Commission granted market authority to the generics company

Sandoz to produce a follow-on to Pfizer's recombinant growth hormone (Tucker, 2007). Shortly thereafter, Sandoz was also approved to produce erythropoietin-α (EPO) in Europe. However, it may not be as easy as it looks. Dutch scientists have found that aggregates due to formulation changes of EPO, sold as Eprex in Europe, were responsible for an immunogenic reaction that triggered severe side effects (Louet, 2003) suggesting that it is possible for biotech companies to provide superior performance through product differences.

Current competition even exists within in the same indication among differing products using disparate mechanisms for treatment. The TNF blockers Enbrel (etanercept), Remicade (infliximab) and Humira (adalimumab) are each used in the treatment of rheumatoid arthritis. The amount and frequency of dosage combined with efficacy and side effects are often factors that determine patient use. Because these products are often used interchangeably, they also compete on perceived quality. If a physician feels less comfortable prescribing a therapeutic that contains proteinacious particles, although equally efficacious, the particle free drug will maintain a competitive advantage.

2.6.3. Cost of Noncompliance

A noncompliance occurs when a pharmaceutical product deviates from the cGMP regulations set forth by the FDA. The regulations include requirements for methods, facilities, and controls used in manufacturing, processing, and packing of a drug product (FDA Office of Pharmaceutical Science, 2008). Divergence from these standards creates serious financial risk linked to patient safety, FDA fines, increased internal resources, patient trust and lost revenue. Although oversimplified, the path of noncompliance could include the following. A defective product leaves the manufacturer and is delivered to a physician whom observes a small white particle in the vial. The physician reports the inferior product to the company which is in turn required to report to the FDA. The product is returned to the manufacturer and internal resources are used investigate the reasons for noncompliance both in the laboratory and the manufacturing facility. The patient and/or physician may no longer trust the product and choose to switch to a competing product. In the worst case, the patient could be delivered the product and suffer a severe reaction causing the patient or family to take legal action. The patients' best interests and the extraordinary risk of regulatory noncompliance drive pharmaceutical companies to meet

regulatory guidelines. For this reason, products that do not comply due to particle related issues are indisputably scrapped, yield is compromised and for the most part, risk is avoided. However, because it is possible for a proteinacious particle to form after inspection or to pass inspection, it is in the firms' best interest to thoroughly determine all factors that could cause protein particles to form to more completely address both risk to the patient and the financial security of the firm.

3. Potential Approaches to Address Protein Particle Formation

As biopharmaceutical pipelines continue to fill with the promise of therapeutic proteins, companies will be challenged to develop and manufacture at a higher standard of quality consistent with the growing body of product knowledge. Advancements in analytical characterization enable biotech companies to provide more product characterization information than ever before. Improved characterization, although constructive to the body of knowledge, is not always seen positively and has made it apparent that protein therapeutic products are more heterogeneous than previously thought or reported (Carson, 2005). Regulatory bodies are expected to increase characterization requirements and standards as more information becomes available. Protein particle formation is an element of heterogeneity that continues to confound product stability, efficacy, or the risk of immunogenic side effects. Regulations are designed to deliver safety and efficacy to patients within a given risk/benefit ratio given the seriousness of the illness. Therefore, aligned incentives and collaboration between the regulatory bodies and biotech companies should exist to create the most value for patients.

The youth of biotech companies and their novel products present a growing challenge for regulatory bodies. In the space of protein particle formation, regulators are learning along side the companies themselves. As such, biotech companies (particularly those with distinguished experience) have the resources to significantly contribute to the body of knowledge that will enable the industry to further develop. For this reason, biotech companies can work to increase the knowledge base needed to influence regulatory bodies and cooperatively maintain extremely high standards of safety and efficacy as a function of the risk/benefit ratio. This can only occur with a collaborative relationship between companies and regulatory bodies. Until that day, companies must use resources both to meet current regulations as stated and to influence the regulatory bodies to adopt updated regulations more appropriate to improved technology. The following sections are designed to focus on efforts that can address current regulatory standards as written (product, process and organizational changes) and those that can potentially influence the regulatory environment (industry standardization and collaboration).

3.1. Meet Regulatory Requirements

3.1.1. Change Product or Process

From the initial stages of research to the final delivery of the therapeutic protein to patients, there are many steps that are designed to ensure product quality and homogeneity. The elements that influence, impact or detect protein particles can be divided into Molecular Design, Formulation, Manufacturing Process, Material Interaction (including container) and Inspection. Although oversimplified, these steps are outlined below. Molecular Design includes research and development leading to the creation of a stable biologically active therapeutic molecule. Formulation begins once the molecule is created and serves to incorporate the protein into a stable solution with excipients to be capable of delivering it to the patient while maintaining the integrity of the protein through expiry. The Manufacturing Process involves the steps necessary to create, purify and transform the protein into a deliverable product. Material interaction overlaps heavily with the Manufacturing Process as it includes the materials the product interfaces with during these processes, but also includes interaction with the material in the storage container. Inspection is one of the many steps to confirm product quality before release. The framework below uses each of these core functions to describe product or process changes capable of addressing regulatory requirements. Related efforts across the industry are reviewed in each of the defined categories.

3.1.1.1. Molecular Design

Once a therapeutic molecule is discovered, it can be manipulated to generate candidate molecules superior to those initially created. Site specific modifications in biotechnology products may introduce new properties, such as improved stability, or new traits, such as drug binding and transport (Lundblad, 1997). Site specific modifications are complex and range in abilities to change the many properties of a protein. Modifications may include a single amino acid change, a whole polypeptide substitution or a combination of both. They can alter the active site, binding affinity, and even protein-protein interactions. Achieved through both chemical modification of the protein itself and mutagenesis of the DNA that codes for the protein, the combination of these techniques may provide an even better strategy than either alone (Lundblad, 1997). The use of the glycoconjugate Poly(ethylene glycol) $(PEG)^2$ is a popular

example of a modification that has been successful in altering the stability of therapeutic proteins including reduced antigenicity (Delgado, 1992) and increased bioavailability (Herman, 1995). Additional protein conjugates and other modifications including covalent cross-linking, have created therapeutic derivatives with enhanced therapeutic effectiveness (Hashida, 1994) and that have also been used to stabilize antibodies (Goldberg, 1991).

By applying a similar methodology, changes in highly aggregation-prone regions and stabilizing interactions could help prevent aggregation and increase long term stability. When studying pathological conditions associated with protein aggregation, Chiti et al. (2003) found that changes in aggregation rates can be attributed directly to the intrinsic effects of the amino acid substitutions on the process of self-assembly and identified mutations in which the aggregation rate could be perturbed significantly. By predicting aggregation based on physicochemical properties of polypeptides and modifying targeted amino acids, Fowler et al.(2005) created variants of the hormone calcitonin that showed significantly reduced aggregation propensity while maintaining or even increasing potency. Modifications that disrupt hydrophobic patches in highly aggregation prone regions, while avoiding changes to residues thought to be important in biological activity, may permit a rational and robust design strategy for additional therapeutic proteins. Other increased kinetic stabilizing options may include introducing hydrophobic mutations, disulphide bonds, salt bridges and metal ions at the protein surface to stabilize and rigidify regions involved in local unfolding (Machius, 2003; Wang, 2004). As one of the earliest steps in the development of a therapeutic protein, molecular design modifications offer the opportunity to proactively improve a molecule using better defined metrics for success.

3.1.1.2. Formulation

After a therapeutic protein has been discovered and produced, several challenges confront pharmaceutical scientists in the formulation of that protein into a stable solution. Formulation desires to protect against the threats of shock, shear/shaking, light, air interface, temperature and pH as a function of protein concentration and time. Formulation types are selected to maximize the pharmaceutical quality of the product depending on the protein stability, clinical needs, and pharmaceutical acceptability (Smales, 2001). Successful formulation of proteins depends on a thorough understanding of their physicochemical and biological characteristics, including

chemical and physical stability, immunogenicity and pharmacokinetic properties (Frokjaer, 2005). Although the complexity of formulation science is too exhaustive for the purposes of this discussion, it can have significant impact on the chemical and physical stability of the protein and potentially leads to aggregation and ultimately precipitation (Cleland, 1993).

Surfactants lower the interfacial tensions between liquids and are often added to protein solutions to prevent physical damage during purification, filtration, transportation, freeze-drying, spray drying, storage and delivery (Kerwin, 2007). For example: Polysorbates are amphipathic, nonionic surfactants commonly used in the formulation of protein biopharmaceuticals (Nema, 1997). Polysorbates are both biocompatible and excellent stabilizers against surface adsorption, but still not a silver bullet. They must be used with caution since under some conditions their degradation can lead to chemical modification of proteins and interactions with some proteins may lead to changes in conformation structure with unknown consequences (Kerwin, 2007; Katakam, 1995). The development of additional surfactants may prove successful in creating more robust formulations, and thereby increasing manufacturing flexibility while preventing the phenomenon of protein aggregation.

3.1.1.3. Manufacturing Process

The manufacturing process and formulation are tightly linked and highly dependent on protein stability. Before a protein is formulated, there exists a complex process of cultivating cells and then extracting and purifying the highly sensitive proteins of interest. Factors such as heat exposure, shaking, shearing, freezing, drying and even filtering can individually or collectively damage the protein during manufacturing. pH adjustment and buffer systems as well as protein concentration (Narhi, 1999) and ionic strength (Wang, 1999) have been found to be crucial parameters in controlling protein aggregation during thermal treatment. Additionally, a variety of surfactants have proven successful in protecting proteins by accumulating competitively with proteins at hydrophobic surfaces/interfaces and/or binding directly to proteins (Wang, 2005, Webb, 2002). Surfactants and excipients have also been used to protect against protein aggregation during freezing and drying. Although aqueous solutions are the preferred choice for both convenience of use and ease of manufacturing, a lyophilized formulation fosters long-term stability of proteins that are not stable enough to fill the required shelf life in aqueous solutions

(Smales, 2001). Aqueous and lyophilized formulations are an example of manufacturing decisions determined by protein stability that substantially alters downstream manufacturing processes, convenience and cost. Although too great to cover in depth for the purposes of this work, other manufacturing focused areas of interest could include Ultra Filtration/Diafiltration, nominal filtration, and other interactions causing product stress such as temperature, shaking and shearing, and freezing.

3.1.1.4. Material Interaction and Delivery Device

Parenteral solutions are not the solitary concern of regulatory bodies; materials and particularly delivery devices are in contact with both blood and the solution and are therefore essential to consider when discussing particles. Protein pharmaceuticals come into contact with a variety of materials during manufacturing; however, the focus of this section is the material interactions of delivery devices, including, but not limited to syringes, vials and stoppers. The individual components of a delivery device may incorporate parts made form multiple forms of metal, glass or polymeric material, which may each contribute some variable level of unique particles (Barber, 2000). Silicone oil is used in syringes and stoppers to enable smooth delivery of the solution to the patient by providing lubrication during the delivery step. It has long been suspected to induce protein aggregation. It is suggested that the oil may have direct effects on intermolecular interactions responsible for protein self-association through interaction with protein surfaces or indirectly through effects on the solvent (Jones, 2005). Tungsten is used in fabrication of the syringe needle placement cavity and presents a similar complication. It has been found to leach oxides that cause reactions with protein therapeutic products causing them to aggregate (Rosenberg, 2006). Delivery device research receives less attention, but is necessary for a holistic approach. A thorough understanding of the formulated protein product with the device should include all possible device material interactions as well as device handling variables including long term storage, shock, agitation, pressure, etc.

3.1.1.5. Inspection

Inspection of final product is one of the last steps before drug product is released to the market. It includes the subvisible inspection by microscopy or light obscuration and visible inspection by manual or automated means. Inspection is tightly linked with characterization and in great need

of improved technology (subvisible inspection discussed in greater detail in Chapter 2.5 and Chapter 4). Visible inspection is focused on detecting both cosmetic defects and visible particles. Manual visible inspectors are trained and certified to inspect particular products and differentiate between foreign and proteinacious particles. Because inspection methods are subjective, it is increasingly important for visible particle inspectors to be well trained in both particle detection and characterization. If a product is determined by the manufacturer to naturally contain visible proteinacious particles at a level acceptable by the FDA, visible particle inspectors are challenged to distinguish proteinacious particles more scrupulously and selectively pass them through inspection as satisfactory product. Because standard protein particle training sets are difficult to assemble, inspection experience becomes a more essential factor. Approval of units containing typical levels of visible proteinacious particles, exhaustive training, and methodical inspection may provide a solution for yield losses in which a particle free solution is not possible and solutions containing trace levels of these proteinacious particles must be determined to be safe for patients without impact to efficacy during clinical trials.

3.1.2. Organizational Changes to Leverage Knowledge

The exploration of biology, chemistry and the human body by academic research institutions and private and public companies continues to create a massive base of valuable knowledge. Leveraging knowledge creation, strategic collaborations and joint ventures between academia and firms allow organizations to capture the value being created through managing the body of knowledge. Biotech companies, often functionally organized, run the risk of creating functional islands of information. As a developing science-based industry, there is no superior example with proven performance over time to act as an example similar to that of Toyota in auto manufacturing (Pisano, 2007). Practices in knowledge sharing, management and integration, both horizontally and vertically, remains a work in progress for sustainable performance. Pisano argues that "a*s the scientific knowledge base of drug R&D broadens, the challenges of integrating the relevant pieces become even more difficult and important. To perform well, the sector requires appropriate mechanisms for bringing together and integrating the right mix of cross-disciplinary talents, skills and capabilities...the sector also requires micro-organizational mechanisms for creating truly integrated problem solving and avoiding islands of specialization*". In the case of protein particles, it is apparent that the solution will not lie within

a single functional area or scientific expertise. From early Research and Development to the final shipment of a commercialized product, and many development steps in between, removing protein particles will require a holistic approach.

3.1.2.1. Proactive versus Reactive Resource Allocation

The extended timeline for pharmaceutical development presents a challenge for related developmental resource allocation. Assuming that resources invested earlier in the development lifecycle are proactive in addressing a problem, while those allocated later are reactive, a biotech firm can create a strategic advantage by optimizing resources by a function of risk and payoff. Proactive resource allocation in the biotech industry, although possibly more impactful, adds an extra layer of complexity. The earlier a product lies in the development lifecycle, the more likely it is to never make it to market. As a molecule passes from preclinical tests to Phase I, II, and III of the commercial development, the likelihood of the molecule continuing to the market increases significantly. The early investments must therefore be weighed against the outlying payoff if the molecule is in fact successful. The balance of risk and cost, Figure 6, may change depending on the progress, including timely regulatory filings, of the development lifecycle. Early investments are riskier because the molecule may fail, but eventually lower cost if the molecule is successful. The opposite also applies.

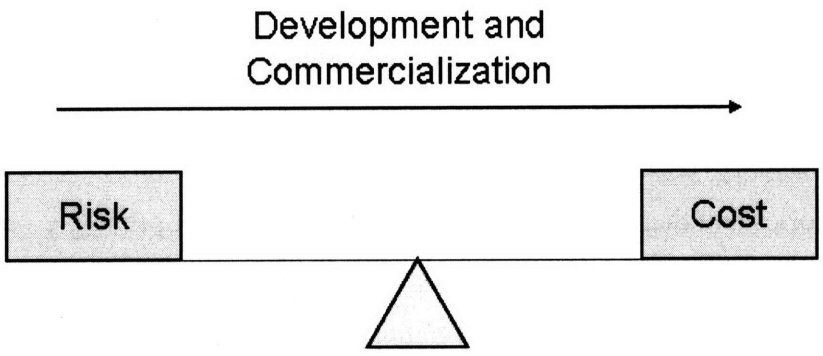

Figure 6: Drug Development Risk and Cost Tradeoff

The framework used in this thesis to analyze a potential approaches to address protein particles was created and presented to correlate to the developmental time frame. Molecular design is one of the first steps after a target or molecule is identified while inspection is one of the final steps after the molecule has been manufactured and determines its release to the market. The steps in

between (formulation, material interactions, process development) can exist in parallel, and although not to scale, also correlate to the development timeframe. Different functional groups are roughly responsible for each of the defined categories and are also an example of the potential to create knowledge silos. Each group proposes projects that consume resources while generating necessary knowledge for regulatory requirements as well as academic interests. Assuming that the objective is to maximize the value of the firm, projects that have the greatest impact should also receive the highest priority.

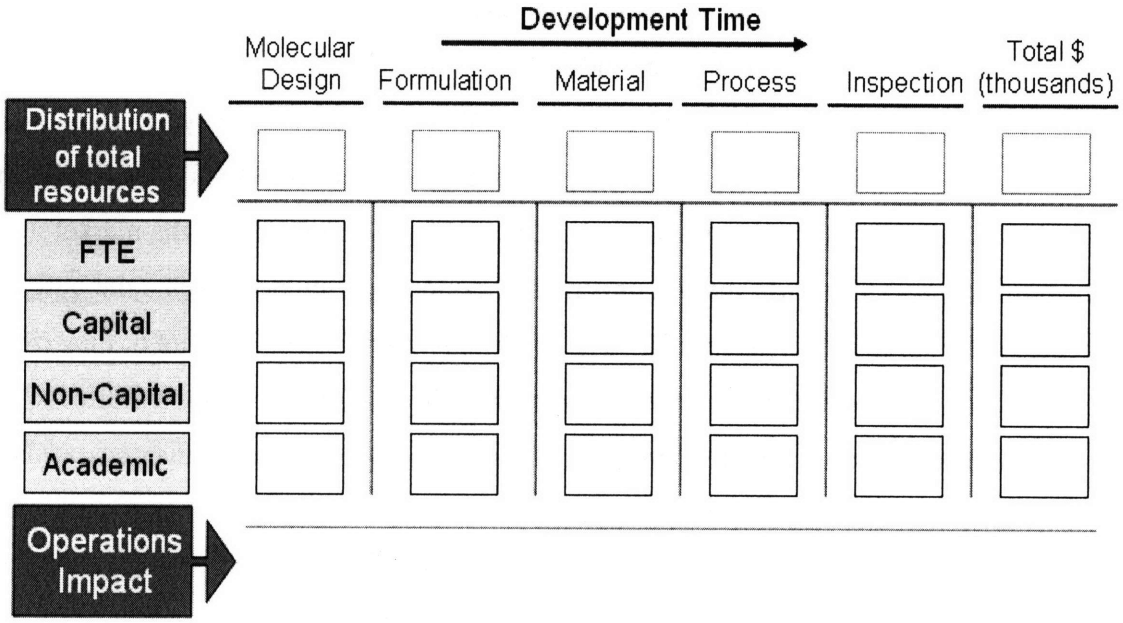

Figure 7: Project Dashboard

Figure 7 was created to visually communicate the allocation of resources to each of the framework categories and therefore demonstrate the distribution of proactive versus reactive resources as well as project prioritization by impact. Resources were calculated using project proposals and generally rough estimations of requirements. For this model to be valuable, the impact is both an important and extremely difficult portion to quantify. To complete the exercise for the purposes of protein particle mitigation, calculation was limited to the value of finished goods yield improvements. Other valuable calculations could include aspects such as material usage, labor and infrastructure requirements.

3.1.2.2. Project Mapping and Testing Integration

To capture value from a holistic approach, it is necessary that functional specific information is effectively shared across all functions tied to a common goal. As each functional group initiates a project related to particles, it is important for efficient collaborative purposes that the project members be connected to other researchers that have had or will have some involvement in a similar project. Without an accurate understanding of both ongoing and completed projects, results and implications, and the value of follow on studies, inefficient resource allocation is inevitable. Implementation of cross functional particle project teams is one sample example of a holistic approach that can address this risk.

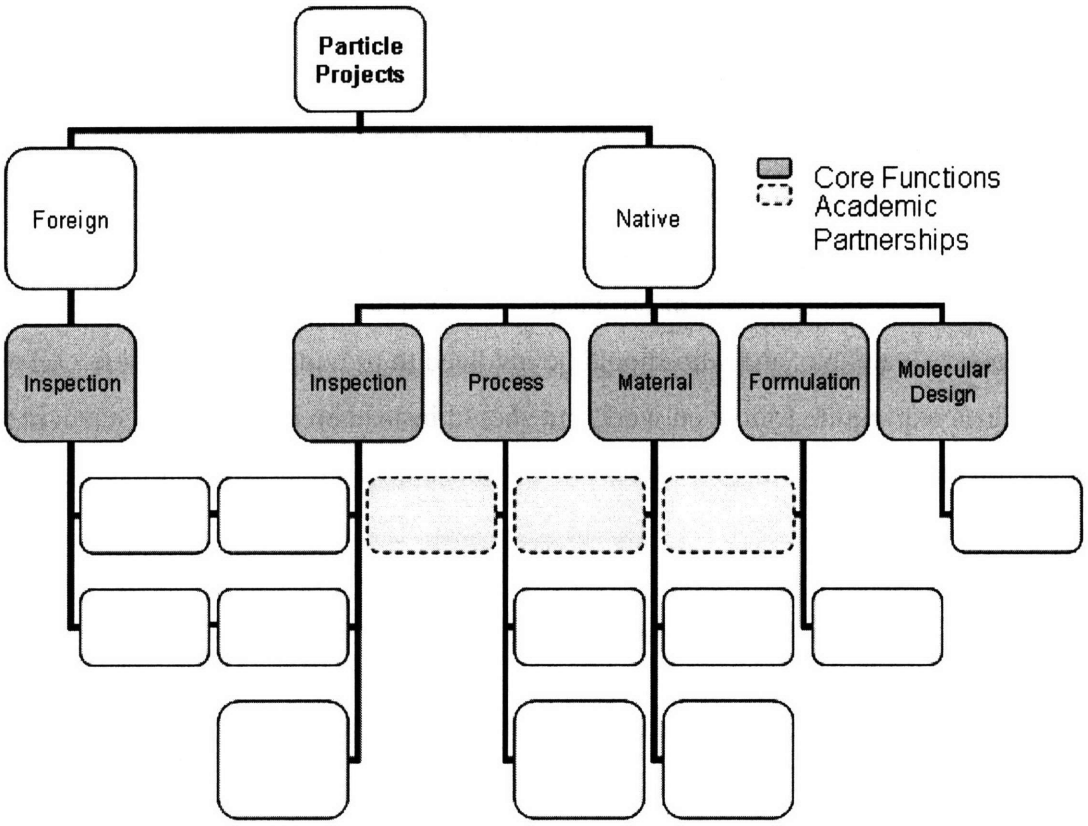

Figure 8: Research Project Map

Figure 8 is an exhibit of a project map created for particle related projects. Because the emphasis of this work is on proteinaceous particles, the foreign heading is included to show that projects related to foreign particles could be either linked or overlapped with those proteinaceous. Similar to the Project Dashboard, the project headings are divided by relevant functional groups

involved in protein particles. Particle related projects and involved researchers are organized under each core functional group responsible for the project (as determined by resource allocation) and divided into categories of internal resources and academic partnerships. These categories are separated because typically, projects through academic partnerships are more theoretical by nature and vary in output expectations, but may provide less calculable value. Access to resources and ease of communication also differs between these groups. By mapping each of the projects, researchers and managers have a resource to understand ongoing work outside a narrow scope, determine gaps, and collaborate when necessary. It is assumed that the researchers have a form of communication (meetings, email, etc.) and an information sharing system in place to record information that together makes knowledge sharing possible.

By managing these projects holistically, it is possible to collectively set expectations and deliverables, track progress, and measure value upon completion. Additionally, best practices can be determined and shared across platforms. Necessary resources, either a cross functional team or project management support, should be determined by the total workload and desired levels of integration. In the interest of efficiency, it is then possible to determine if follow on work is necessary, valuable, and who should be involved to provide the most value. Likewise, if the project does not require follow on work, but should instead be installed as a standard test in development and manufacturing, resources and outputs are well understood for successful future inclusion.

3.2. Influencing the Regulatory Environment to Change

The novelty of biotechnology breakthroughs and its applications create a challenge for regulatory agencies and biopharmaceutical manufacturers alike. Industry and regulatory collaboration presents an opportunity for pharmaceutical manufacturers to innovate superior manufacturing and quality processes.

3.2.1. Process Analytical Technology and Quality by Design

Biopharmaceutical products are created through a combination of tightly controlled manufacturing processes and end-product characterization. Because completely characterized

products are not always possible given available technologies, manufacturing processes also define the end product (Krull, 2006). The Process Analytical Technology (PAT) Initiative intends to drive collaboration between the FDA and pharmaceutical manufacturers to understand and control manufacturing processes. PAT is only briefly covered in this text, but is defined by the FDA a "*a system for designing, analyzing, and controlling manufacturing through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process materials and processes with the goal of ensuring final product quality*" (FDA Office of Pharmaceutical Science, 2008). Biopharmaceutical companies are encouraged to better characterize both products and processes to increase flexibility and trust between manufacturers and regulatory agencies. It is logical to assume that the increase in analytical tool demand from both organizations will drive growth and innovation. It is the responsibility of the firm to strategically use these tools to increase trust, continuously improve processes, and prevent rejects, scrap, and re-processing. The Quality by Design (QbD) element of PAT stipulates that key performance parameters are postulated early in the development process. The designed product and process should be robust for these parameters (Woodcock, 2004). Using these principles, product quality and performance should be achieved and assured by the design of effective and efficient manufacturing processes and product specifications based on mechanistic understanding enabling continuous improvement and "real time" assurance of quality (Berridge, 2004). Because the path to achieve these principles is not entirely known, manufacturers have the opportunity to be an even greater part of its development.

3.2.2. Industry Collaboration and Standardization

An important aspect of collaboration with regulatory agencies includes collaboration and standardization within the industry. By sharing non-proprietary knowledge and collaborating on issues related to regulatory oversight, biotechnology firms have the potential to amplify the value of the industry. As the path to achieve PAT and QbD is still being determined, each firm has product and process experience capable of collectively paving the road. Proteinacious particles are arguably a challenge for any firm producing high concentration protein therapeutics. Industry collaboration creates a single voice to best determine the process specifications and product design criteria that will ensure safety to patients and trust in the regulatory partnerships.

4. Case Study in Improving Analytical Characterization

The nature and complexity of biological products has hindered comprehensive characterization, which differs greatly from small molecule pharmaceutical products. However, increasing instrument capabilities, computing power and regulatory expectations to better understand products is causing biotechnology companies to invest in additional technologies. The goal of this chapter is to compare emerging tools and technologies in development with the current compendia in both the visible and subvisible ranges. The review will include practical considerations and anecdotal information from technicians and researchers familiar with the equipment in addition to equipment manufacturers' claims.

4.1. Analytical Characterization Tool Review

The aim of this section is to investigate tools and technologies focused on the range from 1 μm to 2 mm. To best understand both the capabilities and limitations relative to the purposes of therapeutic protein development, structured interviews were conducted with the principle users of each of the tools. The interviews were designed to identify parameters including precision, accuracy and artifacts as well as operating parameters such as throughput, sample manipulation and ease of user interface. Although some of this data was in a quantifiable form, most elements were subject to the user and the substance (varying products) being analyzed. Although not a principle user, the author offers anecdotal insight based on operating experience as well.

Hiac/Royco Liquid Particle Counting System

The history, theory, capabilities and limitations of light obscuration were discussed in detail in Chapter 2.5.1. The Hiac/Royco Liquid Particle Counting System a commonly used tool (mentioned in USP guidelines) for light obscuration particle analysis in the pharmaceutical industry and is pictured below in Figure 9 (Hach Ultra, 2008).

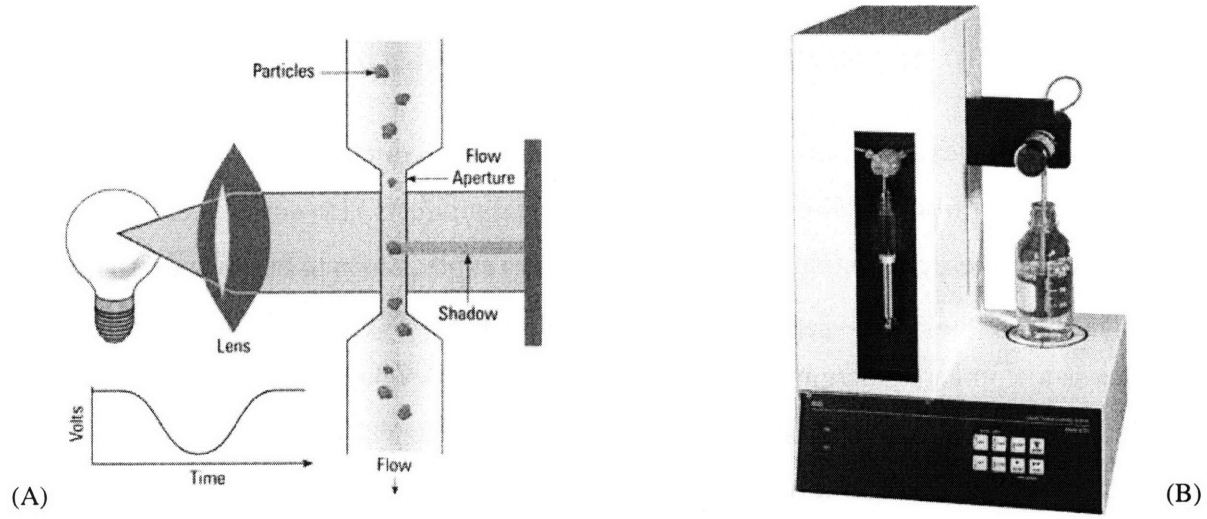

Figure 9: (A) Principle of Light Obscuration. (B) Hiac 9703.

Micro-Flow Imaging

As advances in detection technologies and analytical methods have emerged, BrightWELL Technologies Inc. has combined microscopy, digital imaging, fluidics and image analysis to automatically enumerate and characterize (in terms of size, shape and transparency) particulate matter in liquid samples (Brightwell Technologies, 2008). Micro-Flow Imaging (MFI) uses flow microscopy technology and operates by capturing images of suspended particles in a flowing stream with both a high (14X) and low (5X) magnification option. The images for analysis are selectively captured by the user, as predetermined by a set of criteria, and stored for future analysis. The chosen number of images to be captured does not impact the particle count. The sample volume in each frame is defined by the flow cell geometry which enables the MFI to detect approximately 80% (low magnification) and 50% (high magnification) of the passing volume. Both high and low magnification cannot be used at the same time. The MFI features sample introduction gravity-assisted or by aspiration methods and draws volume using a peristaltic pump.

The flow images are seen in real time on the screen while the software uses an algorithm to calculate particle size as a function of distorted pixels. The software creates an initial histogram with a distribution of particles sizes as chosen by the user ($\leq 0.25\mu m$ increments in size). However, the stored images can then be sorted by multiple criteria including aspect ratio,

diameter, size range, etc. for additional characterization. To optimize the view, flowing images can be seen in both binary and grayscale form. Images can be stored as TIFF or BMP depending on desired resolution and storage space. The options described above are significant in providing the user with increase flexibility to more fully characterize a variety of products.

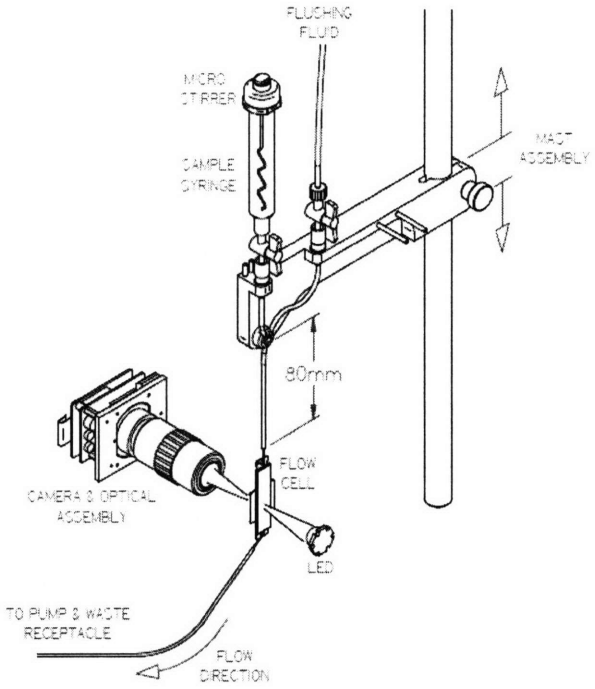

Figure 10: BrightWELL DPA 4100

Figure 10 depicts the apparatus where sample is added to the sample syringe with an optional micro-stirrer (Brightwell Technologies, 2008). The sample then passes through the flow cell where the camera and optical assembly detect particles and record images.

Advantages

Although not a complete solution, the MFI offers a number of advantages that enable more complete characterization of protein therapeutics. The chief advantages are outlined below.

- Ability to capture and store images of particles while in concentrated solution
- Low flow rate reduces shear forces and the risk of particle fragmentation
- Option to fill particle images from outline that appear semi-transparent enabling a size to be determined from the total area

- Software characterization features capable of filtering and sorting data by morphological parameters and creating histograms, trend charts, and scatter plots.
- Greater maximum particle concentration detection (275,000 particles/mL at 2.5μm)
- Increased sensitivity in comparison to light obscuration
- Larger magnification range in comparison to light obscuration
- Results are less dependent on particle shape or material type

Disadvantages

The MFI technology is new to biotechnology and currently under rapid development. Although some of the disadvantages are outlined below, it may be possible that they also exist in light obscuration, but are only now being visualized. The disadvantages include those experienced by Amgen researchers and may differ from the manufacturer's claims or specifications. Because the technology is relatively new to Amgen, it is possible that some of the disadvantages could be reduced or removed through protocol optimization.

Instrument Related

- Precision and accuracy drop for smaller particles under low magnification
- Fluid transitions of differing conditions cause visual streamlining disruption on top and bottom of flow cell
- Miscibility issues may lead to potential miscounts and generation of air bubbles
- Flow cell and optimized illumination of the flow cell are highly influenced by the type of solvent used and may require destruction of sample for optimization without analysis
- Flow cell cleaning and optimization as well as miscibility issues can impact protocol and cleaning procedures which significantly increase testing time
- Particulates or water spots can become permanently embedded in flow cell. Excessive buildup introduces miscount risk and requires replacement of flow cell
- Large sample requirement (manufactures specifications differ from current protocol which requires ≥5mL of sample)
- Image capture and storage requires non-trivial amount of space
- Operations, cleaning and data analysis requires significantly greater periods of time than does light obscuration
- Risk of sensor drying out (may permanently render flow cell unusable)

- Dirty sample tube (dried residue)
- Particle trap tube clogging (prevents constant flow)

Sample Related

- Air bubbles in samples
- Aggregate creation by erratic motorized stirring
- Residue carryover in syringe or stir rod
- Sampling error
- Subjective interpretation of image results

Manual Microscopy with Particulate Filter Analysis System

While manual microscopy has been historically used for subvisible particle detection, the Particulate Filter Analysis System (PFAS) by Clemex attempts to improve the test by integrating a protein specific dye and automation software. The system presents a visualization improvement to known technology and image software capable of narrowing the measurement to particles of interest. As a result, the technician can categorize or eliminate an object by any shape, size, color or texture attribute.

First, a protein sample is filtered through a specific filter paper (varying pore sizes are available) followed by a 15 minute soluble protein specific dye that binds protein and changes it to a purple color. The filter is then placed under the microscope and, using multiple magnification options (dependent on the microscope type), the PFAS can distinguish between protein and non-protein particles with the help of the protein specific dye. Software options allow for a variety of analyses and data on a per particle basis. However, at the current stage in development, the software and analyses are manually intensive and incredibly low throughput. Although the setup and operating times are relatively low, the analysis cycle time is much greater and prohibitive of high throughput analysis. Additional research is needed to understand the complete capabilities of the system.

Advantages

- Differentiation between protein and non-protein particles
- Air bubbles and dissolved gas do not skew data

Disadvantages

- Subjective analysis and interpretation of results
- Filtration pressure may cause some particles to pass through the filter or alter the native structure found in solution
- Complex software and incomplete automation result in low throughput of analysis
- Filter imperfections
- Results are highly dependent on uniform lighting across the filter

4.2. Impact of Method Variability

An often overlooked element of analytical tools, particularly those designed for multiple functions, is the impact of variability of both the sample type, sample preparation and the operating parameters. Although the key capabilities and limitations have been discussed, the operating parameters that make such results possible have been neglected. The capabilities listed above are a combination of both user experience and manufacturing claims. When a user experience differed from a manufacturer's claim, it was determined to be the actual capability based on the method and product type. From a product perspective, the precision and accuracy may vary greatly depending on the type of solution that is being measured and its properties. For the user, software configurations of both the MFI and LO tools allows the user to manipulate settings that include the number of runs and run volume and even run speed. Depending on the limits of the tool, modifications to these settings without a clear understanding of their experimental implications (such as the limit of detection or limit of quantitation) could have a significant impact on the validity of the results. Cleaning, calibration, and tool preparation present similar challenges.

More alarming than the variability in methods, is the inconsistency in scientific reason or method rationale that could generate variability across labs or end-users. The number of light obscuration methods and their underlying principles is a noteworthy example. Two different analytical labs (geographically separated) found two different methods for degassing the samples prior to testing. One lab used a vacuum chamber to remove all air bubbles and dissolved gas from the samples. The other lab found that a sample at ambient temperature for two hours with a rotating magnetic stir bar was able to complete the same task. It could be argued that if the same

outcome is achieved, the variability in the protocol doesn't matter. However, when the developers in the first lab were asked why they chose not to use a stir bar, they responded that a stir bar presented another mode to introduce particles and could also generate protein aggregates through stress prior to measurement. Similarly, when the second lab was asked why they chose not to use a vacuum, they responded by explaining that a vacuum chamber was too difficult to implement into standard manufacturing processes. The underlying reasons for diversity of methods are conflicting and therefore it is natural to question the legitimacy of the results when comparing different protocols. Because the outputs of the analytical tools are highly dependent on the input, it is critically important that all samples are treated similarly so that data is comparable over time, geography and department. Similar inconsistencies existed between two functional groups (geographically co-located) responsible for creating methods for different purposes. If developmental functional groups are not operating using standard protocols, it is possible that the product development could be delayed due to inconsistent or inconclusive results.

4.3. Summary

Table 3 highlights the technical variation of the analytical tools discussed above and the difficulty in comparing each of them. Although Field Flow Fractionation (FFF) falls outside the focused size range of 2μm-200μm, details are included for comparability. They each operate on a diverse set of scientific principles and vary in manufacturer, software and even objective. However, the ability to create orthogonal pathways presents an even deeper understanding of protein aggregates and the development of proteinacious particles in protein therapeutics. For example: experiments designed to compare data between MFI, Microscopy and visible inspection could add strength to each of the tests used individually through cross validation. As emerging technologies continue to become available, it will be increasingly important to determine the potential value of a single tool as it fits with the current portfolio of analytical capabilities. Cross validation combined with gap filling could provide a richer understanding and a higher confidence in the quality of data.

	Light Obscuration	Micro-Flow Imaging	Manual Microscopy	Field Flow Fractionation
Manufacturer	Hiac/Royco Liquid Particle Counting System	BrightWELL	Clemex	Wyatt
Tool	Liquid Particle Counting System	DPA 4100	Particulate Filter Analysis System	Eclipse 2 Separation System
Fluid Speed	10 mL/min	0.2 mL/min	N/A	1mL/min
Manufacturer Range (um)	1.3-400	2.25-400	3-?	0.008-1
Dynamic Range (µm)	2-400	5-400	10-1000	0.002-2
Information Provided	particle concentration	particle images, particle concentration	particle images, particle concentration	relative particle concentraiton
Precision (+/- %)	<5	not available	subjective	not available
Accuracy (+/- %)	5	<10	subjective	not available
Sample Size (mL)	5	5	5	0.02
Sample Tested (%)	100	80	100	100
Sample Prep	vacuum degas	N/A	stain	dilution
Sample Prep Time (min/sample)	120	0	20	10
Run Time (min/sample)	5	15	20	45
Analysis Time	5	20-180	120	39751
Tool Prep Time	10	30-60	20	15-30
Calibration	latex beads	N/A	filter background	BSA

Table 3: Analytical Tool Comparison

5. Focused Investigation of Particle Reduction through Manufacturing Process Changes

The sensitivity of protein molecules and complexity of their production create a dynamic problem for biotech manufacturers. Chapter 3 outlined the areas that should be focused on to remove or reduce particles in human protein therapeutics. Chapter 5 investigates a potential change to the Manufacturing Process by adding an additional step to final purification. This section explores in depth an experiment designed to determine if a process change to bulk drug substance could significantly remove or reduce particles in formulated drug product immediately after the new step and remain effective over time.

5.1. Filtration Hypothesis

The deficiency of analytical tools joined with the complexity of protein therapeutics has inhibited the ability to understand the mechanics and kinetics of protein particle formation. Until recently, the size range slightly above a typical IgG antibody protein dimer (approximately 5-20 nm) ranging to 2μm was especially poorly understood. Without the ability to visibly monitor particle formation, it is unclear how a particle forms and at what point the formation is irreversible. Although future improvements in analytical tools will continue to enhance the clarity of this phenomenon, the limited current state of knowledge and uncertainty in regulation might suggest that one way to reduce the risk of particles is to reduce the presence of particles or remove them altogether.

Filtration remains one of the most utilized techniques to remove insoluble contaminants and purify drug substance. In a typical manufacturing process, product passes through at least five filtration steps in addition to multiple chromatography steps to purify the protein. However, the current filters are not used to remove protein aggregates directly. For example, a viral filter (similar to those used in the experiment) with a nominal pore size of 20 or 50 nm is used to remove potential viruses, but because an additional Ultrafiltraton/Diafiltration (UF/DF) and Bulk Drug Substance (BDS) freeze/thaw step are downstream, the product can experience additional stresses that could then cause it to form particles (or nucleating agents) after the existing viral

filtration. For this reason, it was proposed that a filtration step be completed after BDS Freeze and before Formulation, Fill, Finish steps while maintaining the current upstream viral reduction filtration step.

Protein X is a monoclonal antibody in which data has shown an absence of visible particles upon inspection immediately after manufacturing and filling but presence of visible particulate proteinaceous matter over time. Protein particle formation kinetics suggests that a change in chemical or conformational structure of a single molecule can change its ability to bind to other proteins (described in Chapter 2) is also known as self-association. It was hypothesized that small aggregates (ranging from dimer to small oligomers) act as nucleating agents that, with an altered chemical or physical structure, bind protein product to form larger visible particles over time. Thereby, removal of these small nucleating agents by filtration will reduce the number of particles formed over time. Because aggregates can be created at various points in the manufacturing process, Protein X BDS presents a desirable point to increase manufacturing flexibility. Before entering the final Formulation and Fill, Finish Process, Protein X BDS is filtered through a 0.2 μm nominal pore filter composed of Polyvinylidene Fluoride (PVDF). It is theorized that filters with a smaller pore size will be better at removing the hypothesized nucleating agents. As a result, adding an additional filtration step in the manufacturing process that more completely purifies BDS could prevent or delay particle formation in Bulk Drug Product. The assumptions of aggregation, manufacturing stresses and material interaction suggest that it is still possible for nucleating agents to form in the processes beyond the proposed additional filtration step. However, the point of filtration is chosen for ease of implementation and the probability to reduce or remove the majority of nucleating agents as downstream processes are considered to be less stressful.

5.2. Experimental Design

To determine the growth of particles over time, the experiment was designed to compare standard manufacturing control material to material passed through additional filters of similar composition with decreasing nominal poor sizes at similar conditions. The presence and formation of subvisible particles was then measured immediately upon filtration and again at three time points (approximately 30, 60, and 160 days after filtration). Visible particles were

inspected more frequently. The compendial tools (HIAC and SEC) were used to measure particles and compared to the results of emerging tools (FFF and MFI) of comparable dynamic range capabilities. Replicates of each filter type were created to identify variability in the efficacy of the filters to consistently remove nucleating agents.

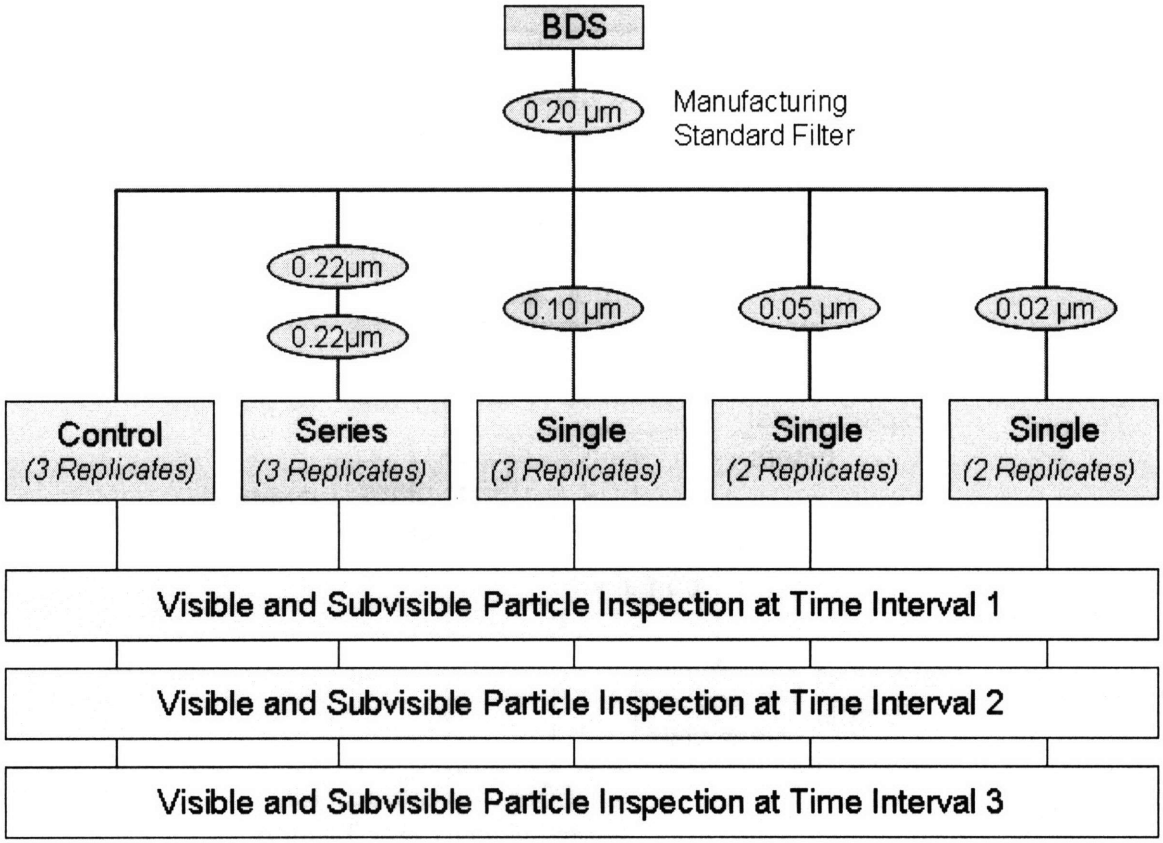

Figure 11: Schematic of Experimental Design

Figure 11 is a visual representation of the design of experiment (DOE). To simulate a proposed change to manufacturing process, we used BDS thawed between 1-14 days prior to filtration. The BDS was passed through a filter of nominal pore size and membrane composition/chemistry as that used in the standard manufacturing process. The material passing only through the manufacturing standard after thaw was used as the control. A 0.22µm filter was used in series to understand the effect of multiple filtration events at the same poor size. The in-series material was collected after the first filtration and then passed again through a new filter of the same lot number. In order of decreasing pore size, material was separately passed once through a

0.10µm, 0.05µm and 0.02µm filter. Only two replicates were created for the 0.05µm and 0.02µm due to limitations of laboratory equipment while triplicates were created for all other filtration types. Figure 12, below, illustrates the filtration process in more detail.

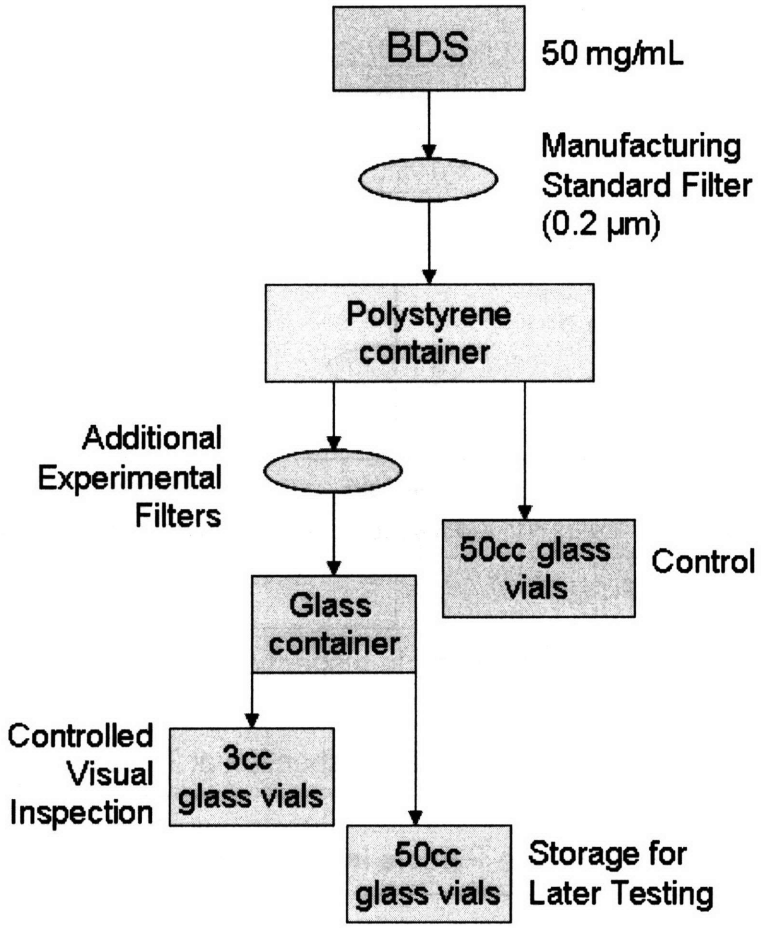

Figure 12: Filtration Experimental Procedure

To ensure that all physical material interaction was free of bacterial contaminants, all glassware, filtering and handling equipment was sterilized prior to filtration. However, the filtering equipment was set up on a bench (not hood) and therefore not entirely free of air contaminants. Because extended material interaction was a consideration, the filtrate was limited to polystyrene and glass for storage. To create similar conditions for each filter type, each day of filtration consisted of a manufacturing standard run followed by the experimental filter completed in the same day. The material was then stored overnight at 4°C in glass containers. The following day, the filtrate material was transferred in a Pilot Plant Fill Finish hood into two 3cc vials per replicate (sealed for non-destructive visual testing) and three 50cc vials per replicate for storage.

Filtrate material to be used for immediate tests was placed into 5cc or 10cc vials. To minimize air contamination, all transfers from storage containers into 5cc or 10cc vials for subsequent tests were completed in the in Pilot Plant Fill Finish hood. All glassware was depyrogenated prior to use.

Visual inspection vials were filled in the Pilot Plant Fill Finish hood and sealed for the duration of the study. The 'visual' vials were stored at 4°C, except during the times of inspection as defined by the standard visual inspection protocol. 3cc vials were chosen for the purposes of highest accuracy and common inspection form for the certified inspector. The visible particle inspector (regarded as one of the most capable and well trained) was held constant throughout the study. Because visual inspection is not destructive, samples were inspected repeatedly over multiple time points. For the first 30 days, the samples were inspected between every 3-6 days.

5.2.1. Filtration Methods and Materials

The following materials were used for filtration:

- Protein X
- Protein X 'PASS' Buffer (sterile, filtered at 0.22μm)
- Millipore pressure vessel (600mL max working volume, polycarbonate and 5L max working volume, steel)
- Pressurized gas source (compressed air)
- Pressure regulator (provided desired pressure)
- Pressure gauges (confirmed operating pressure)
- Balances (0.01g accuracy) assume 1g/L density for filtrate to measure volume
- Polystyrene containers (overnight storage)

Step	Description	Buffer/Solution	Duration
1	Flush the filter with water and remove any air in the lines or filter	Water	≥100L/m²
2	Once steady flow is achieved, measure normalized water permeability (NWP)	Water	N/A
3	Equilibrate filter with buffering solution	PASS Buffer	≥500mL
4	Warm load material to room temperature from 4ºC (storage)	N/A	N/A
5	Filter load material collecting volume measurements with time at a constant pressure	(50mg/mL)	As long as necessary
6	Flush membrane to recover product	Not performed	N/A

Table 4: Filtration Experimental Procedure

Sample Number	Pre-filter	Filter Name	Lot Number	Filter Chemistry	Filter Area (cm2)	Pore Size (µm)	Operating Pressure (psi)	Date of Filtration
1	N/A	Millipore OptiScale	C5MN15604	PVDF	17.7	0.20	10	9/5/2007
2	N/A	Millipore OptiScale	C5MN15604	PVDF	17.7	0.20	10	9/5/2007
3	N/A	Millipore OptiScale	C5MN15604	PVDF	17.7	0.20	10	9/5/2007
4	0.2	Millex GV	R3AN92456	PVDF	3.9	0.22	15	9/5/2007
5	0.2	Millex GV	R3AN92456	PVDF	3.9	0.22	15	9/5/2007
6	0.2	Millex GV	R3AN92456	PVDF	3.9	0.22	15	9/5/2007
7	0.2	Millex VV	R1CN73650	PVDF	3.9	0.10	15	9/10/2007
8	0.2	Millex VV	R1CN73650	PVDF	3.9	0.10	15	9/10/2007
9	0.2	Millex VV	R1CN73650	PVDF	3.9	0.10	15	9/10/2007
10	0.2	Millipore NFR	Sample	PES	13.8	0.05	20	9/10/2007
11	0.2	Millipore NFR	Sample	PES	13.8	0.05	20	9/10/2007
13	0.2	Sartorius Virosart CPV	7000483	PES	180	0.02	50	9/13/2007
14	0.2	Sartorius Virosart CPV	7000483	PES	180	0.02	50	9/13/2007

Table 5: Filtration Experimental Conditions

5.2.2. Practical Implications of Filtration Experiment

Despite efforts to create similar testing conditions for different filter types, it was not possible to maintain consistency along all parameters. As seen in Table 5, the filter chemistry, filter area and pressure varied along multiple filter types. These variations were each due to limitations (either by availability of the filter or physical capability of the experiment) that have subsequent implications. The filter chemistry of the Millipore and Sartorius filters were only available in Polyethersulfone (PES) where PVDF is more typically used in manufacturing processes. For this reason, a PVDF filter was used when available and the PES filters were used as necessary. Different interactions between the protein and surface materials based on chemical and physical parameters (adsorption, electrokinetics, membrane matrix) may introduce discrepancies in the way protein aggregates or other particulates are filtered. However, follow-on experiments would

need to be completed to determine the variation and significance of differing filter membrane composition/chemistry.

The filter area represents the amount of space available for the material to pass through. All material was passed through a filter with an area of 17.7cm^2 at 10 psi to create a low stress environment that returned the material to a state as close to manufacturing standard as possible. A portion of the initial filtrate was saved as the control and the remainder was used for the experimental filtrations. Scale-down 3.9cm^2 filters were available and usable in 0.20μm and 0.10μm filters. The scale-down 3.9cm^2 0.05μm filters were available, but incapable of passing the required volume upon preliminary experiments due to low flux rates (ml/cm2). The Sartorius 180cm^2 filter differed in shape and dimension and achieved increased surface area through a chamber filter design. As the surface area of the filter decreased, the same amount of material experienced more stress to completely pass through the filter. Similarly, as the pore size of the filter decreased, the material also experienced increased time to pass. This "pore plugging effect," as a result, required an increase in pressure (house gas) to induce the material to pass through the filter membranes. Unfortunately, the increased surface area was not sufficient to compensate for the change in pore sizes and although the surface of the 0.05μm filter was two orders of magnitude higher, the pressure required a 5x increase from the control but remained below the max pressure as recommended by the manufacturer for each of the filter housings. Although the 0.02μm filters employed a >10x increase in surface area, on average, 1/3 of the protein material was not recovered in the filtrate as measured by reduction in protein concentration after filtration (see Table 6). These results suggest that at least some protein at high concentration filtered through the 0.02 μm filter is maintained at the surface of the membrane due to partial plugging. This hypothesis is further supported by the decreasing flux over time of filtration.

Filter pore size (μm)	0.20	0.22	0.10	0.05	0.02
Average protein lost to filter membrane	0%	1%	-2%	1%	34%

Table 6: Protein Material Lost in the Filtrate

The time to complete filtration is a similarly significant consideration. The 0.02μm filter consumed the most time and ranged from four to six hours to pass approximately 90mL of 50 mg/mL BDS. Although this number is expected to vary depending on the flux, which is a function of the rheology, formulation (excipients), and the product and protein properties, it still presents a significant technical challenge when considering practical implementation of the additional filtration step. Again, the filter area would need to increase in size enough to make the processing time feasible, while minimizing hold-up or dead volumes. Because it is possible that increasing the surface area would not impact the filtration time, additional experiments to determine the effective filtration area and pressure would need to be completed. It should also be recognized that commercial filtration uses tangential flow which minimizes pore plugging. Due to mechanical complexity of the scale-down model, this feature was absent in the experiment.

Lastly, it is prudent to consider the stress of additional filtration on the integrity of the protein itself. As the nominal pore size decreased, the pressure and time required to pass material through the filter also increased. Foaming of the filtrate was observed during the 0.02μm filtration suggesting a factor contributing to additional and possible irreversible degradation of the protein. Further experimentation would be required to determine an optimal filter area and pressure that might eliminate this problem.

5.2.3. Particle Detection Methods and Materials

The methods and materials are described for each of the analytical tests described in Figure 13 below. SEC, LO and Visual Inspection serve as current compendial tests used for soluble (SEC) and insoluble particle detection. FFF provides information in a comparable size range to SEC while MFI is comparable to LO. These tools are currently in development and may serve as future orthogonal tests to provide richer characterization information. SEC and FFF both provide qualitative information strictly in the subvisible region. LO and MFI provide quantitative information in both the subvisible and visible regions while visible tests are performed by manually by human inspectors and provide information exclusively in the visible region.

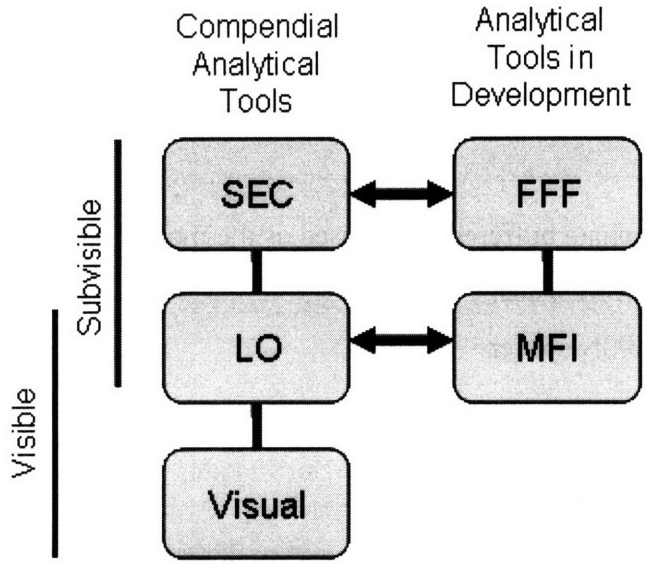

Figure 13: Particle Detection and Characterization Tests Performed

Size Exclusion Chromatography High Performance Liquid Chromatography (SEC HPLC)

The following materials were used to complete SEC HPLC tests at each of the three time points. One operator completed all three time points.

- Protein X
- Protein X 'PASS' Buffer (sterile, filtered at 0.22µm)
- YMC Diol-300 8 x 300mm, 5µ, cat. # DL30S05-3008WT
- Mobile Phase: 100mM Sodium Phosphate, 600mM NaCl pH 7.0
- Agilent 1100 HPLC, Diode Array detector

Conditions

- Room Temperature
- 1 mL/min flow rate
- 20 minutes
- Wavelength = 215 nm
- 10 µL injection volume of 2 mg/mL (20 µg)

Field Flow Fractionation (FFF)

The following materials were used to complete FFF tests at each of the three time points. One operator completed all three time points.

- Protein X

- Protein X 'PASS' Buffer (sterile, filtered at 0.22µm)
- Wyatt Eclipse 2 Separation System coupled with an Agilent 1100 HPLC system
- Wyatt DAWN Heleos MALS detector and an Agilent UV detector set to wavelengths 215 and 280 nm
- Dulbecco's phosphate buffered saline used as the mobile phase (cat# 14190)
- Short channel, 490M spacer
- Large channel, 490N spacer

Conditions

- Room Temperature
- Channel flow of 1mL/min
- Crossflow decreasing gradient from 1 to zero mL/min over 30mins
- Dilutions in Protein X PASS Buffer

Table 7 below describes how test conditions changed across the three time points measured. Variation in the conditions is not expected to have an impact on the accuracy of the data provided.

Time Point	Materials	Concentration (mg/mL)	Total Load (µg)
1	Short channel with a 490M spacer	5mg/mL	10
2	Short channel with a 490M spacer	1mg/mL	10
3	Large channel with a 490N spacer	1mg/mL	20

Table 7: Field Flow Fractionation Test Condition Variations

Light Obscuration

The following materials were used to complete LO tests at each of the four time points. Three operators were used to complete all four time points.

- Protein X
- Protein X 'PASS' buffer
- Particle count control standards – shall contain NIST traceable microspheres which have a certified mean diameter of 15.0µm ± 0.1µm and a concentration above 1000 (#/mL)
- 10cc Glass vials
- Milli-Q filtered (through a ≤ 0.22µm filter) deionized water, 18.2mΩ
- Liquid particle counting system (Hiac/Royco 9703)

- Liquid borne particle sensor (Hiac/Royco HRLD-150)
- 1 mL sampling syringe
- Small sensor probe
- PharmSpec software
- System configuration
    - 10 mL/min flow rate
    - Channel setting (µm): 2, 5, 10, 15, 20, 25, and 50
    - 0 mL tare volume
    - 1 mL sample volume
    - 4 runs per test
    - Discard the 1st run, average the next 3 runs and report (cumulative counts/mL, or particles/mL)
- Vacuum regulator
- Vacuum chamber
- Vacuum pump

Step	Description	Solution	Amount/Duration
1	Vacuum degass samples at 75 Torr	N/A	2 hours
2	Rinse	Milli-Q deionized water	Until 2µm average cumulative particle count ≤1 particle/mL
3	Run standard	Particle count control standards	Single run
4	Rinse	Milli-Q deionized water	Until 2µm average cumulative particle count ≤1 particle/mL
5	Gently hand-swirl vial (15-20 times)	N/A	10 sec
6	Let sample vial stand	N/A	1 min
7	Run sample per protocol	N/A	4mL
8	Repeat rinse between samples	Milli-Q deionized water	Until 2µm average cumulative particle count ≤1 particle/mL
9	Repeat sample run until complete	N/A	Single run
10	Rinse	Milli-Q deionized water	Until 2µm average cumulative particle count ≤1 particle/mL
11	Store probe	70% IPA	N/A

Table 8: Light Obscuration Test Procedure

Micro-Flow Imaging

The following materials were used to complete LO tests at each of the four time points. Two operators were used to complete all four time points.

- Protein X formulated in 'PASS' buffer

- Particle count control standards – shall contain microspheres which have a certified mean diameter of 15.0μm ± 0.1μm and a concentration above 1000 (#/mL)
- DPA4100 Particle Analysis System (BrightWELL #BP-4100-SYS) equipped with DPA4100 Software Release 6.9 and an automated peristaltic pump
- 400μm Flow Cell, 1.6 mm wide
- 10 mL Silanized Syringe
- Stopcocks
- Detergent (50% diluted)
- Isopropyl Alcohol
- 10cc Glass vials
- Milli-Q filtered (through a ≤ 0.22μm filter) deionized water, 18.2mΩ
- Pipettes and appropriate tips

Step	Description	Solution	Amount/Duration
1	Degas at Ambient Temperature	N/A	2 hours
2	Flush Flow Cell	Detergent	5mL
3	Flush Flow Cell	Milli-Q deionized water	20-25mL
4	Run blank from clean syringe	Milli-Q deionized water	Until 2μm average cumulative particle count ≤10 particle/mL
5	Run standard	Particle count control standards	Single Run
6	Rinse and optimize	filtered protein stock	5mL
7	Run sample per protocol	N/A	5mL, 1mL tested
8	Repeat rinse and optimization between samples	filtered protein stock	5mL
9	Repeat sample run until complete	N/A	5mL, 1mL tested
10	Flush Flow Cell	Detergent	5mL
11	Flush Flow Cell	Milli-Q deionized water	20-25mL

Table 9: Micro-Flow Imaging Test Procedure

Visible Inspection

Replicates of each sample (26 total vials) were created one day after filtration. 1.7mL of material was transferred to 3cc vials in the pilot plant formulation and fill hood and then protected from light and stored at 4°C. Due to the subjective nature of the visual inspection test, a single certified inspector was used to control for variability. The following materials and methods were used for visual inspection.

- Protein X

- Inspection Station (Custom-built with light source including a 200 foot-candle fluorescent light with a black and white background)
- 3cc vials and rubber stoppers

Visible particulates were characterized as foreign or protein-like particulates based on the following general characteristics:

Protein Particle

- Small in size (approximately 0.1-0.4 mm) (Typically but not always)
- Transient (may appear and disappear)
- Color: white translucent and/or "C" shaped jelly-like structure
- Fibrous or amorphous in nature
- Neutrally buoyant in solution

Foreign Particle

- Often larger than 0.1mm
- Color: typically observed are white, yellow, red, blue, black, and brown
- Hard or elastomeric
- Metallic or fibrous
- More difficult to re-suspend
- When swirled usually settle down to the bottom of the vial or syringe (when denser than formulating) or float up (when less dense than formulation)

Step	Procedure
1	The vial samples were taken out of the refigerator and allowed to equilibrate to room temperature for approximately one hour. Each vial was then cleaned from the outside using Kimwipes wet with IPA solution to remove any residue or condensate on the outside.
2	Each vial was slowly inverted and slightly rotated to distinguish between the minor defects on the outside of the vial and the moving particulates
3	Each vial was held at the top part of the inspection station (approximately 6-8 cm below the light) for optimal viewing
4	The approximate distance from the inspector's eyes to the vial was approximately 7 to 10 in.
5	The approximate exposure time for the actual inspeciton of each vial was about 1 min.
6	Each vial was inspected utilizing both the black and white background of the inspection station (following steps 1-5)
7	The total number of particulates were counted and documented
8	Once complete, the syringes were covered and placed back into the refrigerator at 4ºC

Table 10: Manual Particle Visible Inspection Characterization Method

5.2.4. Particle Detection Results

Particle detection test were completed for 3 time points using SEC HPLC and FFF and for 4 time points for LO and MFI. As a non-destructive test, visible inspection was completed every 3-5 days for the first month to closely monitor kinetic growth.

5.2.4.1. Subvisible

The subvisible range is defined as particles with a length or diameter below 125µm extending to the protein monomer. Both the SEC and FFF capabilities are limited to the subvisible region while the LO and MFI tools are capable of detecting both subvisible and visible particles. LO and MFI data are reported as cumulative particle counts ≥5µm. Although the capabilities of the tools range into the visible region, results are presented in the subvisible section for simplicity. SEC and FFF both report relative amounts of particles or particulate species as a percent of the total product analyzed while LO and MFI report data in particles/mL. The results from the subvisible tests are provided below.

Size Exclusion Chromatography

SEC tests were submitted to an SEC technical lead and completed for three time points ranging between 1-9 days (Time 1), 35-43 days (Time 2), and 146-154 days (Time 3) after filtration. The data was analyzed by measuring the relative area of the monomer peak in comparison to aggregate peaks and low molecular weight (LMW) species. The main peak represents the amount of monomer while the aggregate peak represents those species larger than monomer (ranging from dimer to small oligomer) and the LMW peaks represent the presence of proteinacious material smaller than the monomer.

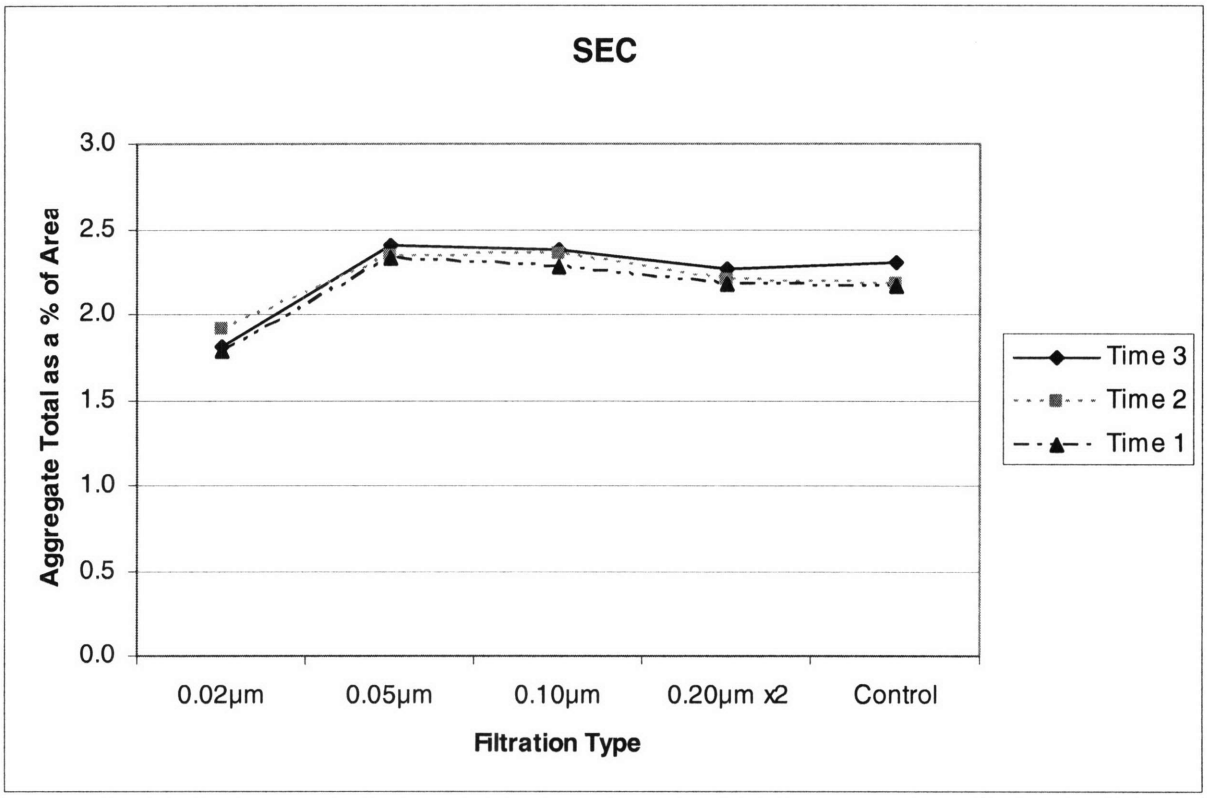

Figure 14: Aggregate Detection by SEC

Figure 14 exhibits the amount of total aggregate detected in samples from each filtration type over time. The trend across filtration types remains consistent, while it is clear that the 0.02µm filter has the lowest percent of total aggregate. All other filtration types (0.05µm, 0.10µm and 0.20µm in series) appear to have relatively little impact when compared to the control.

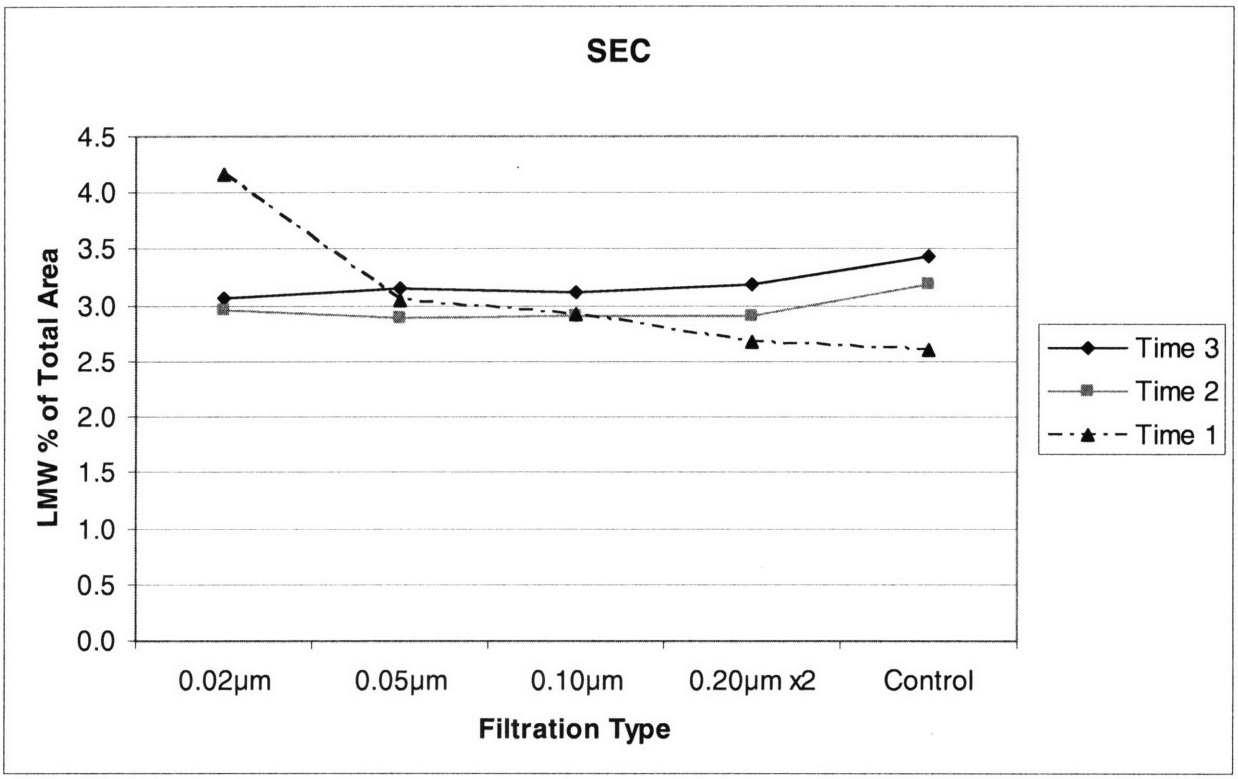

Figure 15: Low Molecular Weight Protein Detection by SEC

Figure 15 shows the amount of LMW species measured over time. Here the trend is less clear than in the case of total aggregates. At Time 1, there is a much higher presence of LMW species in the 0.02µm filter and a lower presence in the control.

Field Flow Fractionation

FFF tests were submitted to an FFF technical lead and completed for three time points ranging between 5-13 days (Time 1), 77-85 days (Time 2), and 161-169 days (Time 3) after filtration. Similar to SEC, the data was analyzed by measuring the relative area of the aggregate peak in comparison to the monomer peaks. At Time 1, only one representative sample was chosen from each filtration category (samples 1, 5, 8, 11, and 14). Here, the amounts of monomer and particulates are not directly quantified.

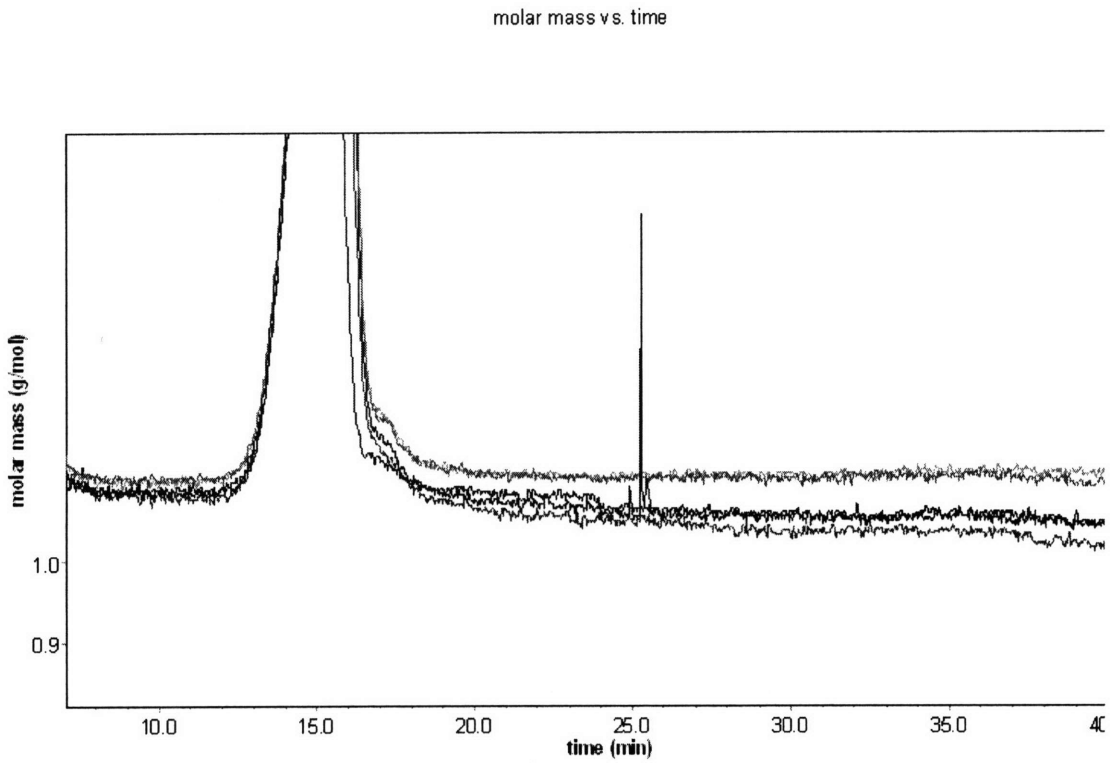

Figure 16: Particle Detection by FFF at Time 1

Figure 16 is a magnified picture analyzing the presence of monomer and aggregate species. For each filtration type, the large peak at 150 min represents monomer while a similarly shaped, but smaller peak after monomer would indicate the presence of aggregates. The spike at approximately 25 min is not an aggregate peak and is instead considered an artifact of the run. The separation of lines as time progresses is considered a drift artifact and is also not significant. The above figure does not point toward the presence of any detectable subvisible particulate

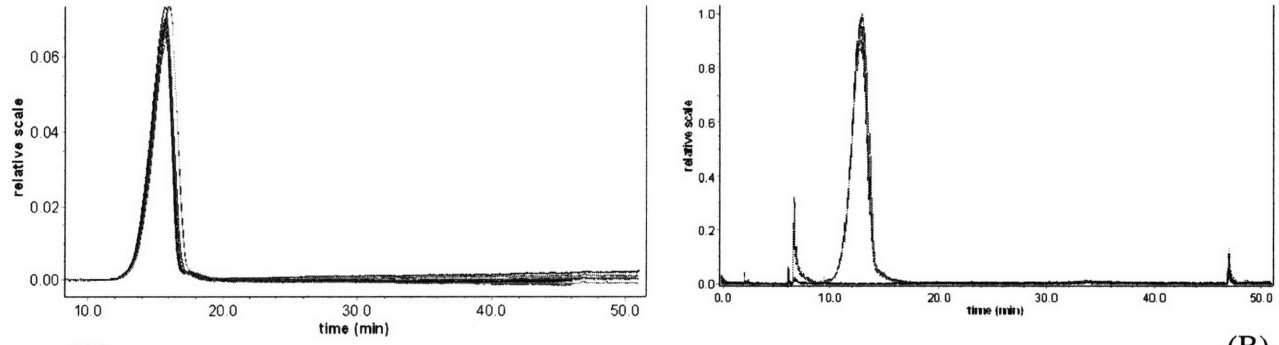

Figure 17: Particle Detection by FFF at Time 2 (A) and Time 3 (B)

At Time 2 and Time 3, representative samples were not used and instead, each replicate per filtration type was run once. Figure 17 shows full scale results from the FFF tests at Time 2 and Time 3. Similar to Figure 16, the large peak is indicative of monomer while a peak thereafter would be the evidence of subvisible particles. A measurable number of particles was not detected by the FFF analytical tool in the range of monomer to 2μm during any of the analyses.

Light Obscuration

LO tests were completed by two operators in the course of four time intervals. Measurements at times 1, 2 and 3 were completed one day after filtration. Measurement at time 4 ranged in 151-159 days after filtration. During a typical LO by Hiac/Royco test, the 1st run is discarded and the 2nd, 3rd, and 4th runs are averaged by the instrument software and recorded as average cumulative counts. However, because an order of magnitude drop in cumulative counts was consistently seen on the Protein X samples (common only to this protein) between the 2nd and 3rd run, only the 3rd and 4th run were used to create the average cumulative counts used in the analysis. Because the 2μm range is near the lower bound of the tool and well below both the USP and European Pharmacopeia reporting requirements (≥10μm and ≥25μm) the results were not used. Instead, average cumulative counts were calculated using particles ≥5μm in size. Acceptable limits for the blank and control were met during each run.

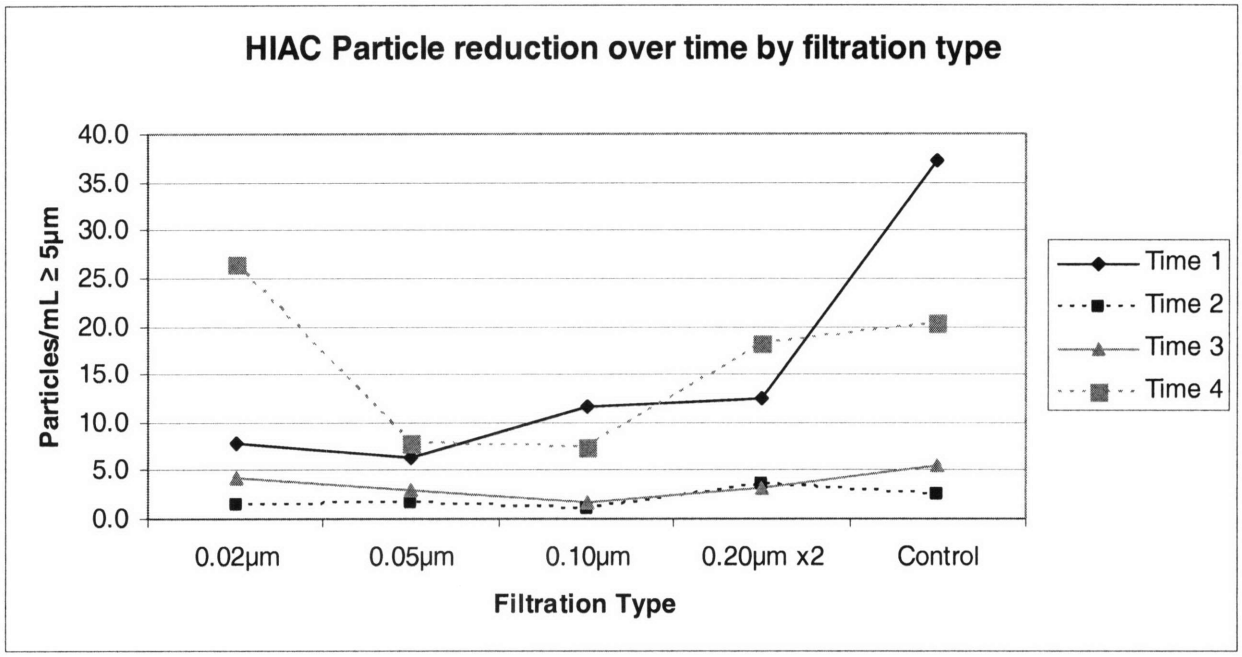

Figure 18: Particle Detection by Light Obscuration

Figure 18 describes the average number of cumulative particles ≥5µm across filtration types. Time 1 shows a trend in removal of particles correlating to the filtration type. As the filter pore size decreases, the average number of particles decreases as well. However, at Time 2 and 3 it is observed that all filtration types show a relatively similar low number of particles and suggest no significant removal by any single type. All counts are lower than what is observed at Time 1. The trend of Time 4 shows an increase in particles from Time 2 and Time 3 across all filtration types. The 0.02µm in series and Control types also amplify by an order of magnitude. The particle counts of the 0.05µm and 0.10µm filter types remain consistently low at each time point and may illustrate some optimal range of pore size capable of removing particles while limiting the amount of stress on the protein.

A one-way analysis of variance of cumulative particles by filtration type was completed at each time point. The results are summarized in the table below.

Time	1	2	3	4
P value	0.1647	0.6641	0.4065	0.1075

Table 11: Analysis of Variance Using LO

Each of the P values is considered insignificant and could easily be this large by happenstance. More data might help to determine significance. Additional statistical analyses were completed to understand the significance of the data and are included in the appendix.

Micro Flow Imaging

MFI tests were completed by two operators for four time intervals ranging in 12-20 days (Time 1), 35-37 days (Time 2), 74-76 days (Time 3) and 152-160 days (Time 4) after filtration. According to the protocol, sample material was used to purge the system followed by a single run measuring 1mL of material. For the same reasons as outlined in the light obscuration results, the data was analyzed by calculating the cumulative particle counts ≥5µm averaged across filtration types of the same pore size. Acceptable limits for the blank and control were met during each run.

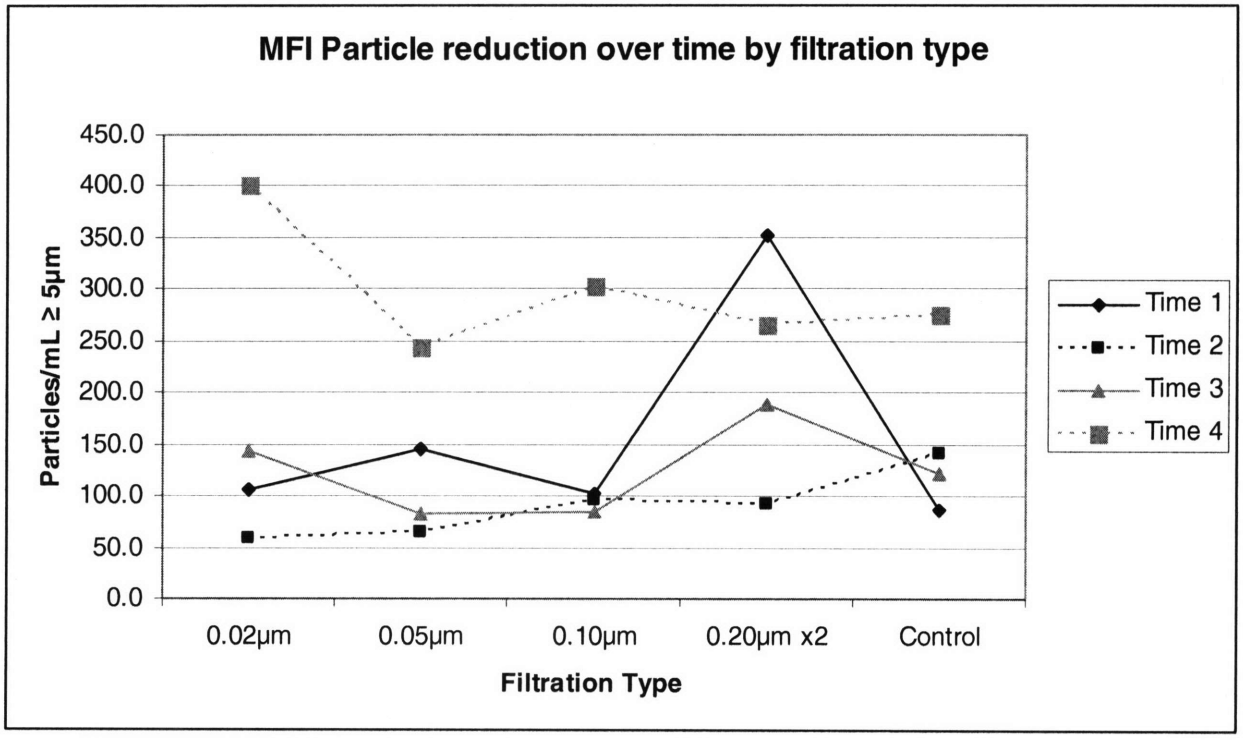

Figure 19: Particle Detection by MFI

Figure 19 describes the average number of cumulative particles ≥5µm across filtration types as detected by Micro Flow Imaging. Although the trends are not as clear as those demonstrated by LO, a similar pattern is detected at Time 4. The 0.02µm filter type makes a significant leap from

the levels detected at Time 3 while all filtration types are >25% lower than the 0.02μm filter type. At Time 4, all other filtration types appear to have relatively the same impact on reducing particles over time. The 0.05μm and 0.10μm filters exhibit less variability in lowering the particle counts which may also be evidence of an optimal filter pore range. It is also important to note that the MFI particle count data is an order of magnitude higher than that collected from LO.

A one-way analysis of variance of cumulative particles by filtration type was completed at each time point. The results are summarized in the table below.

Time	1	2	3	4
P value	0.5187	0.335	0.1424	0.4843

Table 12: Analysis of Variance Using MFI

Again, each of the P values is considered insignificant and could easily be this large by happenstance. More data might help to determine significance. Similar to LO, additional statistical analyses were completed to understand the significance of the data and are also included in the appendix.

5.2.4.2. Visible Inspection

Visual inspection is a subjective test and difficult to maintain consistency even for a single inspector let alone the possibility of inspector to inspector variation. The descriptions of particulate matter were collected and the results divided into fibrous and round particles. Despite efforts to provide a clean environment free of foreign particles, the process development lab and pilot plant fill finish conditions are not maintained to manufacturing standards and therefore it is not possible to guarantee a particle free environment during experimentation. If the inspector was able to quantify the number of particles, the total number was included in the data set. If a particle was considered "foreign" by the inspector, the counts were not included in the data defined to detect protein particles. However, fluctuations in the data suggest that particles could have been counted as both foreign and proteinacious depending on the day of inspection. For this reason, it is both difficult and somewhat unreliable to quantify the data. The number of

particles (round and fibrous) were summed and averaged across filtration types for each time interval after filtration.

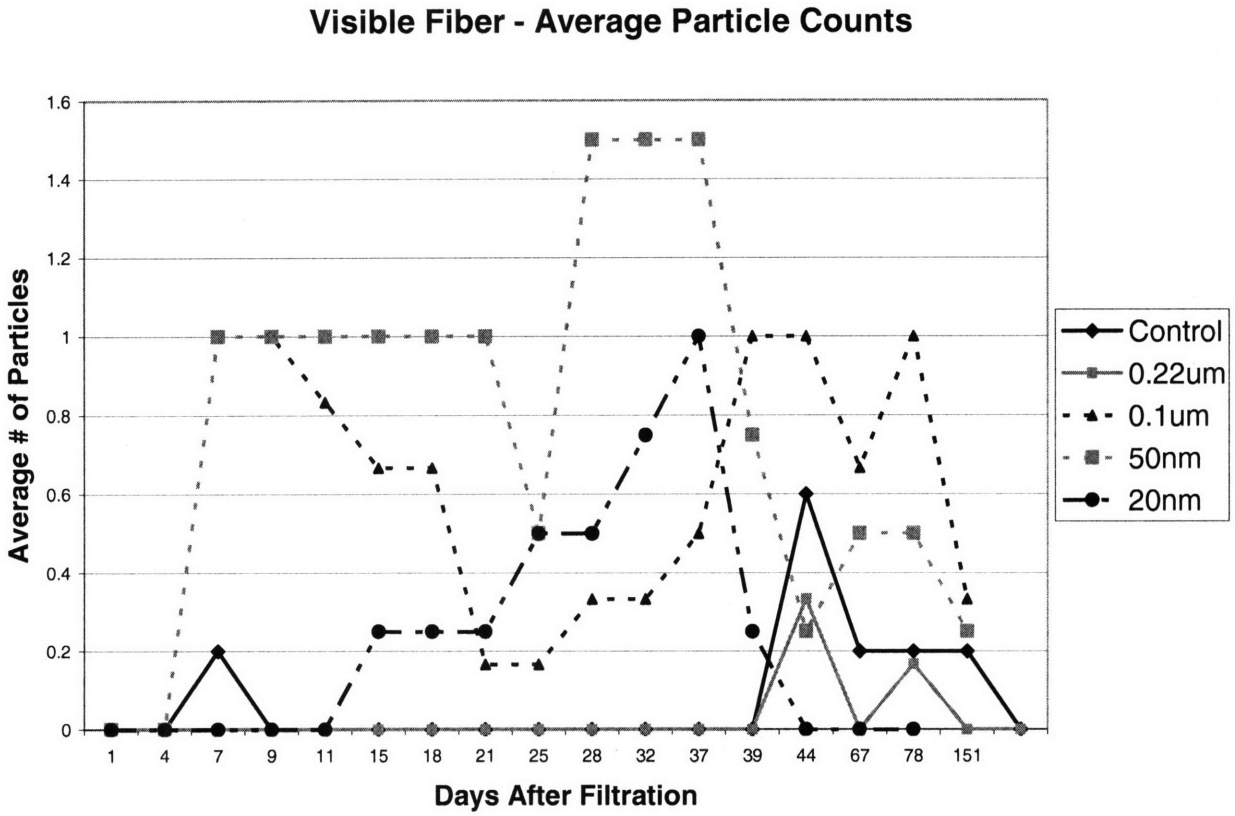

Figure 20: Average Visible Fibrous Particles

Figure 20 above displays the average number of fiber-like particles in each filtration group over time. Each number on the X axis represents an inspection point in days after filtration. Because the samples were controlled (never opened after transfer into vials), the observed variability can be attributed to the inspector and the behavior of the particles. The numbers do, however, remain relatively low and the particles show no significant increases over time. Contrasting with the data from the subvisible detection, the highest visible fiber-like particle counts are seen in the 0.02µm, 0.05µm and 0.10µm filters and the lowest counts are seen in the Control and 0.22µm in series. However, because these fiber particles are very low overall, the presence of a single foreign fiber counted as a proteinacious particle can significantly skew the data. A similar problem is seen when quantifying the visible round particles with the presence of microbubbles.

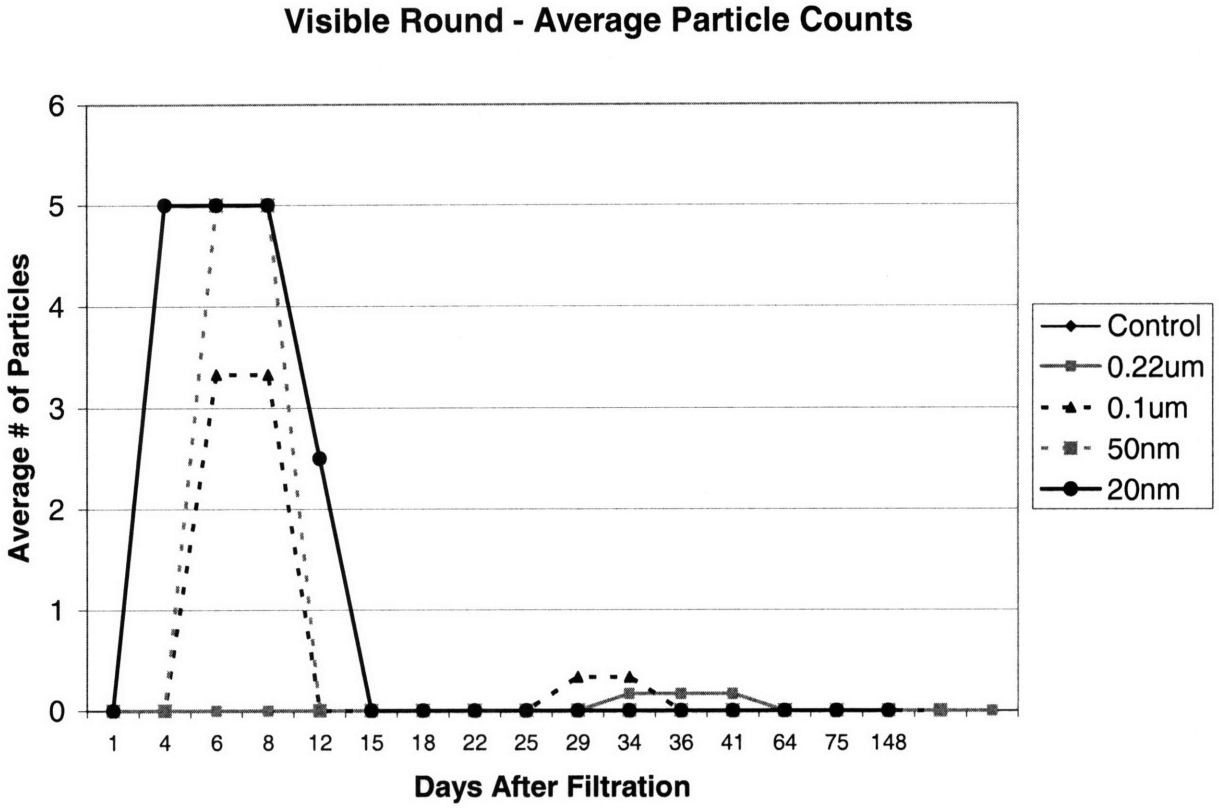

Figure 21: Average Visible Round Particles

Figure 21 shows the number of visible round particles presented in the same way as the fiber-like particles. Similar to the fiber-like counts, the 0.02µm, 0.05µm and 0.10µm filters exhibit the lowest average counts while the Control and 0.22µm in series are almost always lowest. However, it is important to note that corresponding to the significant drop in round particles, all samples are described as having "many microbubbles" present. The particle descriptions reported abruptly change from "nothing observed" (9/14/07) to "many microbubbles" (9/17/07) in all of the samples. The presence of microbubbles may significantly inhibit the ability to see round particles as supported by the maintained drop in round particles 15 days after filtration. It is not clear what could have caused all of the samples to form "many microbubbles" in the three days between inspections as inspection conditions were maintained and the samples remained unopened.

5.2.4.3. Discussion

This experiment was carried out with the intention of determining the ability of filters with nominal pore sizes smaller than those currently used in downstream manufacturing purification to remove or reduce the presence of protein particles both immediately and over time. The data collected, however, does not appear to make a conclusive argument. SEC shows a <1% reduction of total aggregate (oligomers) in the 0.02µm filter type. The general SEC trend suggests that as the pore size decreases, the stress on the protein increases, causing "clippings" or degradation of the protein creating an abundance of LMW species. The later disappearance of LMW species might suggest binding to other proteins in solution leading to the possible formation of particles over time. FFF did not detect a presence of subvisible particles and showed no difference between the control and other filtration types.

LO and MFI both offer the advantage of detecting particles in the native solution while SEC HPLC and FFF require diluted material. The LO and MFI measurements suggest that there may be an optimal range in pore sizes able to reduce larger subvisible particles over time. The 0.05µm and 0.10µm filters appear to consistently maintain low particle counts during the approximately 160 day period. The MFI shows a similar pattern, however all particle counts appear to increase at time 4, particularly the 0.02µm filtered samples. The considerable leap in the number of particles at time 4 of the 0.02µm filtered samples might be indicative of the effects of filtration stress over time. The current implication of an optimal range of filtration pore size is unconvincing, but statistical analyses suggest that more data is needed to make more certain claims. The visual inspection was also inconclusive and seemingly biased by foreign particles and the belated incidence of microbubbles. Additional tests are needed to determine the feasibility of particles to remove or reduce proteinacious particles. However, the tests could be narrowed with the understanding that practical application of the 0.02µm filter to a manufacturing process would require either a sizeable increase in surface area (to be determined by additional experimentation) or and an acceptance of significant material loss; either implication is costly. Even if the filter shows a reduction in particle count, the gained yield would need to offset the material lost in the production process. Additionally, the 0.22µm in series filter could be removed from future tests as it appeared to have no positive impact.

6. Conclusions and Recommendations

Amongst the exciting prospects of the biotechnology industry lie equally novel challenges for its practitioners to address. Unlike other industries, it faces an especially complex product, an extensive development life cycle and strict regulations that help ensure safety to the patients it serves. The nature of these three challenges presents the potential for a firm to create a competitive advantage in the industry. Each challenge is tightly intertwined and their overlap is suggestive of the need for a holistic approach to address the problem. Thorough characterization of the product and processes reduces product risk and regulatory pressure. Organizational optimization reduces the development lifecycle by efficiently sharing knowledge and leveraging the extensive knowledge base.

Proteinacious particles are an example of one of the new challenges facing the biopharmaceutical industry. Chapter 2 presented the observation that not only is it unclear how they arise, but their clinical relevance is not understood and significant gaps exist in the literature correlating side effects with particle size and number. The ability to generate a thorough understanding of the product is a function of the tools capable of visualizing the problem. The mechanisms leading to particles are not entirely understood because it is not possible to track their formation from monomer to visible particle. Chapter 2 explores the taxonomy, kinetics, measurement and regulation of protein particles as they are understood today. Although their existence is not entirely deciphered, their impact on the business is. In addition to increasing the risk of the firm and the safety of patients, protein particles consume development resources and reduce product yields which directly impact the bottom line.

There is an assortment of approaches, some more tactical than others, that a practitioner can use to address protein particles in the overall strategy of the business. Chapter 3 broadly divides potential approaches into those looking to meet current regulatory requirements and those looking to influence future regulation. To meet regulatory expectations, a framework is presented to align functional groups and their processes across the development lifecycle. Alignment and collaboration are necessary to holistically address the problem and utilize the resources of the firm. Ongoing research across the firm and academia is presented to briefly

review some of the potential resources. Within this framework, opportunities exist to align expectations across the functional processes and leverage knowledge generated by both the firm and academic institutions collectively working to advance science and the industry.

Analytical characterization remains one of the chief bottlenecks in the thorough diagnosis of protein particles. The purpose of Chapter 4 is to explore the capabilities and limitations of both current and new innovative technologies to achieve that end. Protein particles exist in a very dynamic range; this section, however, is focused on subvisible particles that are of the greatest interest to regulatory bodies. Here, it is suggested that the individual strengths of these technologies lies in comprehensive and cross validated use.

The further exploration of one tactical approach is the focus of Chapter 5. It is hypothesized that by changing the manufacturing process to include an additional filtration step, particles and there nucleating agents may be reduced or removed altogether. In this experiment, a variety of filters with pore sizes smaller than those currently used at a single purification step are investigated to achieve particle reduction. Both current and innovative analytical tools are used to measure the particles initially after filtration and at time intervals covering a five month period. The results of the experiment suggest that although additional filtration may reduce particles in some optimal range, it is inconclusive and requires more targeted experimentation. It is important to recognize that filtration is only one tactical response in which the need for a more proactive comprehensive answer exists.

The goal of this thesis was to present a framework for biopharmaceutical companies to mitigate the affect of protein particles. From this paper's perspective, it is in the best interest of the firm to address both the current regulatory requirements and proactively collaborate within the industry and with regulatory bodies to change how the problem is thought of in the future. The answer does not lie within a single segment of science or functional process in the industry and therefore requires a holistic approach. While this thesis focuses primarily on a specific line of attack, it is only one piece of the puzzle. As a problem that directly effects the bottom line of a firm, it is imperative to act strategically through organizational optimization and tactically through effective experimentation and project execution that will further the body of knowledge.

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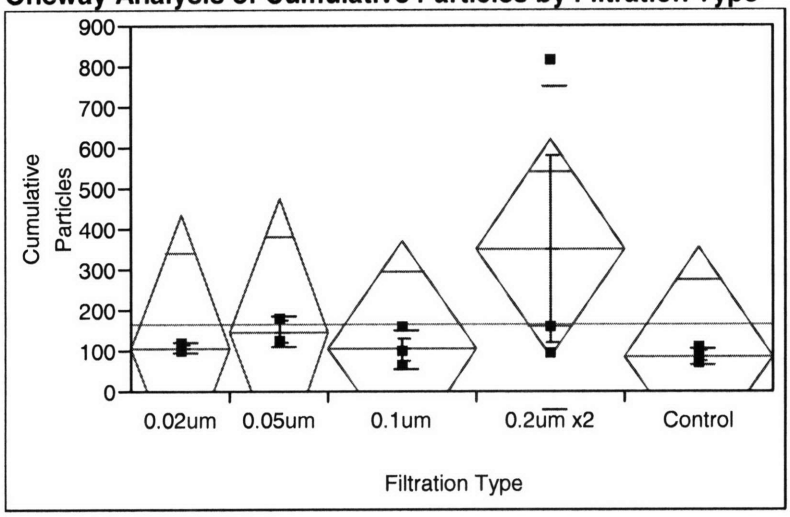
Appendix

T=1

Oneway Analysis of Cumulative Particles by Filtration Type



Oneway Anova Summary of Fit

Rsquare	0.304642

Analysis of Variance

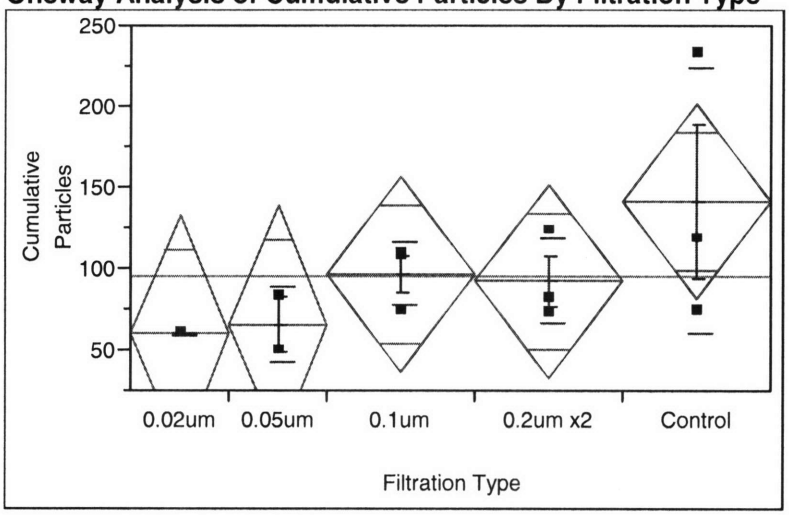
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Filtration Type	4	142666.10	35666.5	0.8762	0.5187
Error	8	325640.67	40705.1		
C. Total	12	468306.77			

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
0.02um	2	106.000	12.728	9.00	-8.4	220.4
0.05um	2	146.000	38.184	27.00	-197.1	489.1
0.1um	3	103.000	48.877	28.22	-18.4	224.4
0.2um x2	3	352.000	399.000	230.36	-639.2	1343.2
Control	3	86.333	20.502	11.84	35.4	137.3

T = 2

Oneway Analysis of Cumulative Particles By Filtration Type



Oneway Anova Summary of Fit

Rsquare	0.401138

Analysis of Variance

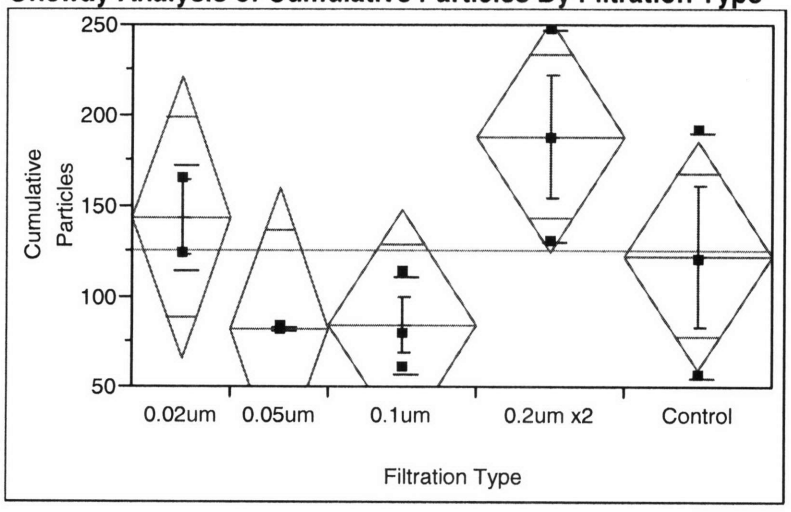
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Filtration Type	4	10824.744	2706.19	1.3397	0.3350
Error	8	16160.333	2020.04		
C. Total	12	26985.077			

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
0.02um	2	59.500	0.7071	0.500	53.1	65.85
0.05um	2	65.500	23.3345	16.500	-144.2	275.15
0.1um	3	96.333	19.3993	11.200	48.1	144.52
0.2um x2	3	92.000	26.2869	15.177	26.7	157.30
Control	3	141.667	82.0995	47.400	-62.3	345.61

T = 3

Oneway Analysis of Cumulative Particles By Filtration Type



Oneway Anova Summary of Fit

Rsquare	0.539098

Analysis of Variance

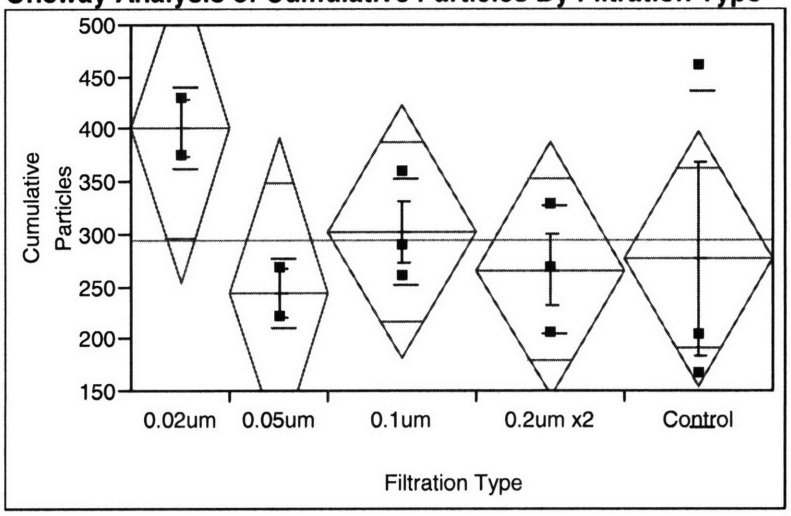
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Filtration Type	4	21347.603	5336.90	2.3393	0.1424
Error	8	18251.167	2281.40		
C. Total	12	39598.769			

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
0.02um	2	143.500	28.9914	20.500	-117.0	403.98
0.05um	2	82.000	1.4142	1.000	69.3	94.71
0.1um	3	84.000	26.8514	15.503	17.3	150.70
0.2um x2	3	188.000	58.5064	33.779	42.7	333.34
Control	3	122.333	67.5302	38.989	-45.4	290.09

T = 4

Oneway Analysis of Cumulative Particles By Filtration Type



Oneway Anova Summary of Fit

Rsquare	0.321611

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Filtration Type	4	31376.077	7844.02	0.9482	0.4843
Error	8	66183.000	8272.88		
C. Total	12	97559.077			

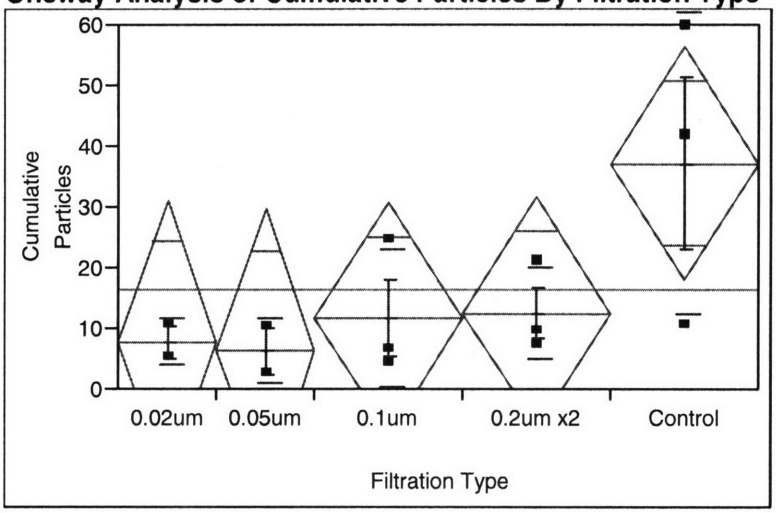
Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
0.02um	2	400.500	38.891	27.500	51.1	749.92
0.05um	2	243.500	33.234	23.500	-55.1	542.10
0.1um	3	301.667	50.895	29.384	175.2	428.10
0.2um x2	3	265.667	60.501	34.930	115.4	415.96
Control	3	275.667	159.788	92.254	-121.3	672.60

HIAC

T = 1

Oneway Analysis of Cumulative Particles By Filtration Type



Oneway Anova Summary of Fit

Rsquare	0.518936

Analysis of Variance

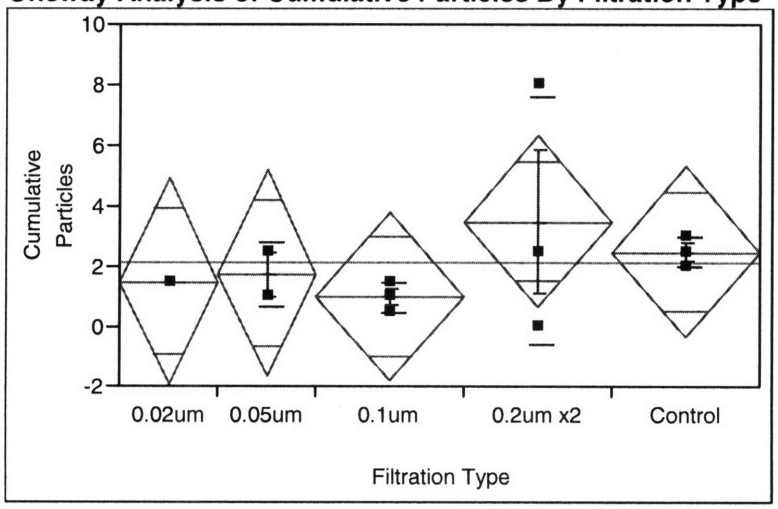
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Filtration Type	4	1762.1859	440.546	2.1574	0.1647
Error	8	1633.5833	204.198		
C. Total	12	3395.7692			

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
0.02um	2	7.7500	3.8891	2.750	-27.19	42.692
0.05um	2	6.2500	5.3033	3.750	-41.40	53.898
0.1um	3	11.6667	11.1841	6.457	-16.12	39.449
0.2um x2	3	12.5000	7.4666	4.311	-6.05	31.048
Control	3	37.1667	24.7857	14.310	-24.40	98.738

T = 2

Oneway Analysis of Cumulative Particles By Filtration Type



Oneway Anova Summary of Fit

Rsquare	0.235136

Analysis of Variance

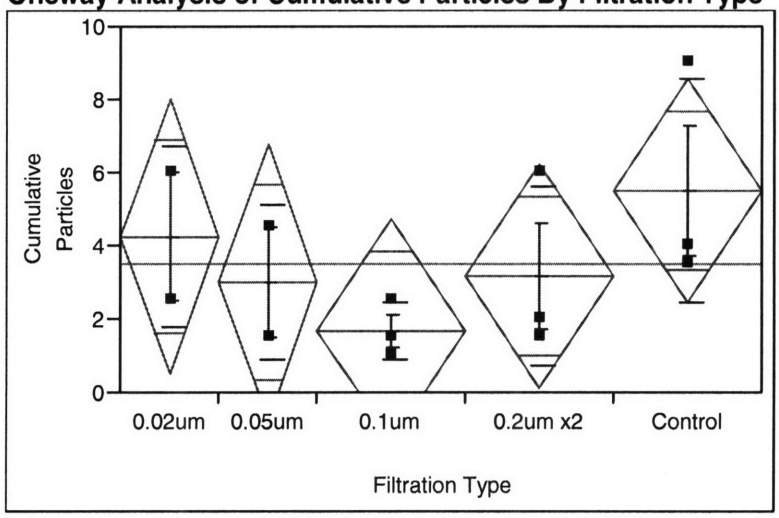
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Filtration Type	4	10.951923	2.73798	0.6148	0.6641
Error	8	35.625000	4.45313		
C. Total	12	46.576923			

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
0.02um	2	1.50000	0.00000	0.0000	1.500	1.500
0.05um	2	1.75000	1.06066	0.7500	-7.780	11.280
0.1um	3	1.00000	0.50000	0.2887	-0.242	2.242
0.2um x2	3	3.50000	4.09268	2.3629	-6.667	13.667
Control	3	2.50000	0.50000	0.2887	1.258	3.742

T = 3

Oneway Analysis of Cumulative Particles By Filtration Type



Oneway Anova Summary of Fit

Rsquare	0.361529

Analysis of Variance

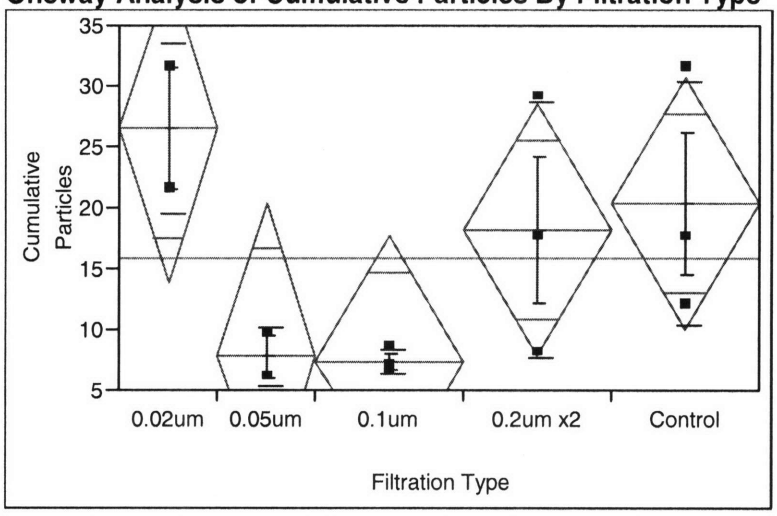
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Filtration Type	4	24.041667	6.01042	1.1325	0.4065
Error	8	42.458333	5.30729		
C. Total	12	66.500000			

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
0.02um	2	4.25000	2.47487	1.7500	-17.99	26.486
0.05um	2	3.00000	2.12132	1.5000	-16.06	22.059
0.1um	3	1.66667	0.76376	0.4410	-0.23	3.564
0.2um x2	3	3.16667	2.46644	1.4240	-2.96	9.294
Control	3	5.50000	3.04138	1.7559	-2.06	13.055

T = 4

Oneway Analysis of Cumulative Particles By Filtration Type



Oneway Anova Summary of Fit

Rsquare	0.575171

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Filtration Type	4	652.0673	163.017	2.7078	0.1075
Error	8	481.6250	60.203		
C. Total	12	1133.6923			

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
0.02um	2	26.5000	7.0711	5.0000	-37.03	90.031
0.05um	2	7.7500	2.4749	1.7500	-14.49	29.986
0.1um	3	7.3333	1.0408	0.6009	4.75	9.919
0.2um x2	3	18.1667	10.5159	6.0713	-7.96	44.290
Control	3	20.3333	10.0540	5.8047	-4.64	45.309